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YEASTS OCCURRING ON APPLES AND IN APPLE CIDER[1]

BY D. S. CLARK,[2] R. H. WALLACE,[3] AND J. J. DAVID[4]

Abstract

A study was made of the predominant yeasts that occur on apples grown in Quebec, and in cider. The yeasts isolated from apples were members of the family *Cryptococcaceae* and of the genera *Candida*, *Cryptococcus*, *Rhodotorula*, and *Torulopsis*. Species of *Candida* were by far the most predominant. The yeasts isolated from cider were representatives of the genera *Debaryomyces*, *Pichia*, and *Saccharomyces*. All the yeasts studied were classified into species of the respective genera.

In recent years many of the predominant yeasts occurring on the surfaces of various fruits have been isolated and classified (2, 6, 16, 17, 18, 21). It seems, however, that very few reports have been published about yeasts indigenous to apples; Lewis (12) isolated *Endomyces mali* from decaying apples; Grosbusch (10), *Torula rubesfaciens* from apple parings; and Lodder and Kreger-van Rij (13), *Candida krusei* from an apple. Information concerning the predominant yeasts occurring in apple juice and cider seems also to be scarce; Osterwalder (19) isolated *Saccharomyces heterogenicus* and *S. microellipsodes* from apple juice; Lodder and Kreger-van Rij (13), *S. elegans;* and Marshall and Walkley (15), several species of *Rhodotorula*, *Saccharomyces*, and *Torulopsis*. Guilliermond and Tanner (11) described two strains of *S. mali* isolated from apple cider; Pearce and Barker (20) studied the yeast flora of sweet and black cider and described 13 species of yeasts.

It seems that a taxonomic study of the yeasts occurring on apples and in apple products is timely; the work reported in this paper was done, therefore, to determine what yeasts predominantly occur on apples grown in the province of Quebec, and in cider.

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Experimental

Methods

Isolations from Apples

Twenty-two samples of apples were collected in October, 1952, at two widely separated places in Quebec. At Macdonald College six samples were gathered randomly from crates containing freshly picked McIntosh apples, and at Franklin Center 16 samples of wild apples were collected from several trees. In each instance sterile forceps were used to carry the apples by their stems to large sterile culture dishes. In the laboratory each apple was quartered rapidly by means of a sharp knife, placed with 400 ml. of water in a Waring Blendor bowl, and comminuted four times for 30 sec. at one-minute intervals. The suspensions were diluted to 1 : 10, 1 : 100, 1 : 1000, and 1 : 10,000 with sterile distilled water; five 1-ml. replicates of all the dilutions were plated with apple juice agar (14). The plates were incubated at 25° C. for four days and then examined for yeasts that appeared to be different from one another. Fifty of the most predominant yeasts were isolated, purified by serial plating, and maintained on slopes of a yeast cultivation medium described by Wickerham (22).

Isolations from Cider

Two 400-ml. samples were taken from the taps of each of two large barrels of apple cider. The juice for this cider had been pressed from a mixture of wild apples, treated with sulphite, and transferred to large barrels where it was allowed to ferment and mature under natural conditions for one year. Ten milliliters of each of the four samples were pipetted into flasks containing 100 ml. of sterile apple juice. After the cultures had incubated for four days 10 ml. of each were used to make 1 : 1000, 1 : 10,000, 1 : 100,000, and 1 : 1,000,000 dilutions with sterile distilled water. One milliliter of all dilutions was plated on apple juice agar and incubated for four days. Ten yeasts that appeared morphologically different from one another were isolated, purified, and maintained on slopes of yeast cultivation medium.

Taxonomy

With a few exceptions, the procedures followed and media used for taxonomic studies were those described by Lodder and Kreger-van Rij (13) and Wickerham (22). A modification of a method described by Wickerham and Duprat (23) was used for the examination of pseudo and true mycelia, blastospores, and arthrospores. Petri plates each containing two glass slides were sterilized by dry heat; Wickerham's yeast morphology agar (22) was poured into one set of the plates until it covered the slides to a depth of 1 mm.; Benham's corn meal agar was used for another set of the plates. When the agar had solidified, cells of the yeasts were streaked in a straight line on the agar over the slides; sterile coverslips were placed over a portion of the streaks and the cultures were incubated for five days at 25° C. The slides were

removed from the Petri plates for observation, the agar removed from beneath them, and the growth occurring under and beside the coverslips studied under a microscope.

Seven different media were used to test for the development of ascospores, namely: carrot slices, Gorodokowa's agar, gypsum blocks, malt extract agar, one-sixth strength tryptone glucose agar, and vegetable juice agar. The tryptone glucose agar was prepared by dissolving 6 gm. of Difco tryptone glucose agar and 11.5 gm. of agar in 1000 ml. of distilled water (9). The sodium acetate agar was prepared as described by Adams (1). A method recommended by Gray (8) was used for staining ascospores. The liquid–culture and auxanographic methods were both used for the carbon and nitrogen assimilation tests.

Results and Discussion

Sixty isolated yeasts were studied. Approximately 75% of those occurring on wild and cultivated apples appeared very much the same; 27 of these were isolated and found to have identical micromorphological and physiological characteristics. These characteristics were unlike those of any previously described species; consequently the yeast was given the binomial *Candida malicola* (4).

The names of the species studied, their origin, and their occurrence are given in Table I. It is interesting to note that all the yeasts isolated from apples were members of the family *Cryptococcaceae*. In addition to their

TABLE I

SPECIES OF YEASTS ISOLATED FROM CULTIVATED AND WILD APPLES, AND FROM APPLE CIDER

Source	Yeasts	No. of cultures isolated
Cultivated apples	*Candida malicola*	21
	Torulopsis famata	3
	Rhodotorula glutinis var. *rubescens*	2
	Rhodotorula muciliginosa	2
	Cryptococcus albidus	1
	Cryptococcus neoformans	1
Wild apples	*Candida malicola*	6
	Cryptococcus albidus	5
	Cryptococcus laurentii	4
	Cryptococcus neoformans	4
	Candida scottii	1
Apple cider	*Pichia membranaefaciens*	2
	Saccharomyces oviformis	2
	Saccharomyces cerevisiae	1
	Saccharomyces steineri	1
	Debaryomyces kloeckeri	1
	Torulopsis candida	1
	Candida mesenterica	1
	Pichia polymorpha	1

inability to form ascospores these yeasts were not able to ferment any of the common sugars. They were classified into seven genera and 16 species by means of the schemes of Lodder and Kreger-van Rij (13). The asporogenous yeasts were represented largely by *Candida malicola* and to a lesser extent by species of *Cryptococcus*, *Rhodotorula*, and *Torulopsis*. The perfect yeasts were represented by species of the genera *Debaryomyces*, *Saccharomyces*, and *Pichia*. *Candida malicola*, *Cryptococcus albidus*, and *Cryptococcus neoformans* were common to both cultivated and wild apples. The perfect yeasts found in cider produced abundant and typical ascospores but only the *Saccharomyces* species were capable of fermenting glucose.

Since 27 of the 50 yeasts isolated from apples were identified as *Candida malicola*, it is very probable that this yeast is indigenous to Quebec apples. This yeast was found again in great numbers on McIntosh apples in 1953.

It is probable that the results of this work do not show all the types of yeasts that are indigenous to apples grown in Quebec. It may be that they represent a group of yeasts that are not easily removed from the surface of apples by weather. It was possible to acquire information about the weekly amount of rainfall from only one area, Macdonald College, where a total of 2.84 in. of rain fell during the first week of October, 1952. Since the apples were gathered shortly after this time, it is reasonable to believe that a great many types of microorganisms could have been washed off by the rain. Marshall and Walkley (14) have shown that rainfall considerably reduces the number of yeasts on apples. They reported that the average numbers of yeasts for the months of September and October were 2934 and 424 per cm.^{2}, respectively; the rainfalls were 1.96 and 6.62 in., respectively. Charley (3) stated that appreciable infection of apples by wild yeasts occurs in early autumn and that a large proportion of those additions to the microflora of apples can be removed by washing. Furthermore, Forgacs (7) showed that rinsing apples in tap water reduced the number of mesophilic organisms from 252 to 13 per 4 sq. in. of epidermal surface.

The fact that three of the species identified in the present work were members of the genus *Cryptococcus* strengthens the belief that the yeasts isolated were in indigenous group. Cells of species of *Cryptococcus* are surrounded by a starch-containing capsule, a characteristic that causes them to clump together and give a positive starch–iodine test (22). It is assumed that such capsules would probably aid yeasts in remaining attached to the surface of apples, regardless of the washing effect of heavy rains.

Certain well-defined species of yeasts have been isolated from bottled cider (20), but no evidence exists to show that those yeasts may be especially associated with certain apples. It is interesting to note, however, that over 50% of the cultures isolated from the cider used in this work were non-fermenters and that all the yeasts isolated from the apples were also non-fermenters. It has been noted elsewhere (5) that the presence of the non-fermenting yeasts in apple juice apparently does not influence the fermenting activity of the *Saccharomyces* species.

Acknowledgment

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A STANDARD INOCULUM FOR CITRIC ACID PRODUCTION IN SUBMERGED CULTURE[1]

By R. Steel, S. M. Martin, and C. P. Lentz

Abstract

A standard vegetative inoculum of *Aspergillus niger* has been developed for the submerged citric acid fermentation of sugar beet molasses. Increasing the ferrocyanide content and the pH of the seed mash, within limits, decreased the rate of development of the mold pellets whereas increasing the spore inoculum increased the rate of development. Using this "standard" inoculum, an average yield of 8.9% anhydrous citric acid (68.5% conversion of available sugar) was obtained in 116 hr. The standard deviation between runs was 4.5% of the mean citric acid yield. An abrupt breakdown of the fermentation process decreased the yield by 50% and increased the standard deviation to 28.5% of the mean yield. The breakdown was overcome by changing the sporulation medium from a chemically-defined synthetic type to a nutritionally-richer type. Results indicate that serial transfer on a synthetic medium weakened the culture and made the fermentation unstable.

Introduction

The production of citric acid by molds, in amounts desired commercially, is not a normal metabolic process. The accumlation of large amounts of citric acid is possible only when metabolism is impaired by toxic substances or deficient media (1, 3, 4, 5, 6). This pathological condition is also reflected by changes in morphology. Karow (4) and Karow and Waksman (5) controlled mold morphology by using a nutritionally deficient medium, while Snell and Schweiger (8) used a deficient medium with ammonium carbonate as the nitrogen source. Clement (1) and Martin and Waters (6) used ferrocyanide treatment of beet molasses to control mold morphology.

Martin and Waters (6) found that good yields of citric acid could be obtained by submerged fermentation of ferrocyanide-treated beet molasses using a pellet-type inoculum. However, the seed cultures were not uniform and the amount of inoculum used was difficult to control. Since their results showed that amount and type of inoculum affected yields, further work was undertaken to develop a method for the production of a suitable standard seed-type. The first section of this paper shows the effect of pH, ferrocyanide, and spore levels on the growth of a "standard" vegetative inoculum of *Aspergillus niger*. The second section reports the effectiveness of this inoculum for citric acid production.

Development of a Standard Inoculum

Methods

Sporulation cultures were prepared from soil stocks of *A. niger*—N.R.C. A-1-233 transferred three times in succession on the synthetic medium of Shu and Johnson (7). About one dozen third-transfers were prepared and when fully sporulated were stored at 5° C. One third-transfer was used

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weekly for the preparation of fourth-transfers which were then used for the production of the inoculum. After preliminary studies, the synthetic sporulation medium was replaced by a molasses medium as indicated below.

To prepare spore suspensions, 10 ml. of a sterile 0.05% aqueous solution of "Aerosol O.T." was added to each spore plate and then the spores were wetted and washed free. The spore suspension was poured off and the plate washed twice with sterile water. The combined washings were made up to 100 ml. and were shaken with glass beads to break up clumps of spores. The number of spores was estimated turbidimetrically, the density having first been related to direct spore counts. The final suspension contained 45 to 55 $\times$ 10^6 spores per ml.

Mash for seed cultures was prepared from well-mixed sugar beet molasses diluted with tap water to about 13% sugar concentration and adjusted to pH 6.0 with hydrochloric acid. Fifteen hundred milliliters of mash containing the desired amount of potassium ferrocyanide trihydrate (added as an aqueous solution) was autoclaved at 15 p.s.i.g. (per square inch gauge) for 25 min. and allowed to cool at room temperature. On cooling, the pH was adjusted to the desired level with hydrochloric acid or potassium hydroxide and 0.5 gm. dibasic potassium phosphate trihydrate per liter was added as a sterile solution. Three hundred milliliter aliquots were dispensed into sterile one-liter flasks, inoculated with the desired number of spores, and incubated at 27° C. on a "Gump" rotary shaker running at 160 r.p.m.

Levels of pH, ferrocyanide, and spore inoculum that produced the desired "standard" type of mold pellets (described below) in 20 to 24 hr. were first determined by trial and error. Thereafter, in studying the effect of varying these factors, one of the initially determined sets of conditions was used as a control and other cultures were assessed at the time the control culture reached the "standard" stage of growth. (For Chatham 1951 molasses, these conditions were pH 6.0, 0.40 gm. ferrocyanide per liter, and 600 $\times$ 10^6 spores per culture.)

To estimate the number of pellets produced, twelve 1 : 100 dilutions were made from pooled cultures grown under like conditions. Duplicate counts were made on each sample by withdrawing 1 ml. of the suspension into a pipette and counting the pellets. While the count was being made the culture was agitated on the shaker.

Results

A complete range of morphological types of mold growth was observed. The extremes of this range were represented by the filamentous type (Fig. 1), showing abundant growth and the retarded type (Fig. 2) showing scant growth. The filamentous type of growth was not considered satisfactory because the quantity of mold for an inoculum could not be estimated by counting, while the retarded type was ruled out by poor fermentation results. The pellet type (Fig. 7) was an intermediate form between the two extremes and was selected as the "standard" because it gave good results in preliminary fermentations and closely resembled the pellet type used successfully by

Martin and Waters (6). The individual pellets were 0.2 to 0.5 mm. in diameter with precipitated matter enmeshed in the pellet center. The lateral hyphae were short, thickened, vacuolated, and granular with short club-like branches. This is the characteristic pathological morphology referred to previously (6). The growth stages of the "standard" seed are shown in Figs. 3 to 8. The dark centers of the mold pellets formed at about 16 to 18 hr., and their formation coincided with a clearing of the mash. The "standard" pellet type had a somewhat retarded appearance in the early growth stages (early prestandard, Fig. 4) but rapidly developed lateral growth. The transition from "standard" to "poststandard" usually took from two to four hours depending on the treatment.

When the pH, ferrocyanide, and spore levels were varied in mashes prepared from Chatham 1951 molasses, the results shown in Tables I, II, and III were obtained. The pellet count under the conditions specified for "standard" seed was 2.9×10^3 pellets per ml. of culture.

TABLE I

EFFECT OF FERROCYANIDE AND pH ON PELLET DEVELOPMENT

(600×10^6 spores per culture)

$K_4Fe(CN)_6 \cdot 3H_2O$ (gm./liter)	pH			
	5.0	6.0	7.0	8.0
0.30	Retarded	Filamentous	Filamentous	Retarded
0.35	Retarded	Poststandard	Standard	Retarded
0.40	Retarded	*Standard*	Prestandard	Retarded
0.45	Retarded	Prestandard	Prestandard	Retarded

TABLE II

EFFECT OF FERROCYANIDE AND SPORE LEVEL ON PELLET DEVELOPMENT

(pH 6.0)

$K_4Fe(CN)_6 \cdot 3H_2O$ (gm./liter)	Spores per culture $\times 10^6$			
	450	600	750	900
0.30	Filamentous	Filamentous	Filamentous	Filamentous
0.35	Prestandard	Standard	Standard	Poststandard
0.40	Prestandard	*Standard*	Standard	Poststandard
0.45	Prestandard	Prestandard	Prestandard	Poststandard

TABLE III

EFFECT OF pH AND SPORES ON PELLET DEVELOPMENT

(0.4 gm. $K_4Fe(CN)_6 \cdot 3H_2O$/liter)

pH	Spores per culture $\times 10^6$			
	450	600	750	900
5.0	Filamentous	Filamentous	Filamentous	Filamentous
6.0	Prestandard	*Standard*	Standard	Poststandard
7.0	Retarded	Prestandard	Prestandard	Standard
8.0	Retarded	Retarded	Retarded	Retarded

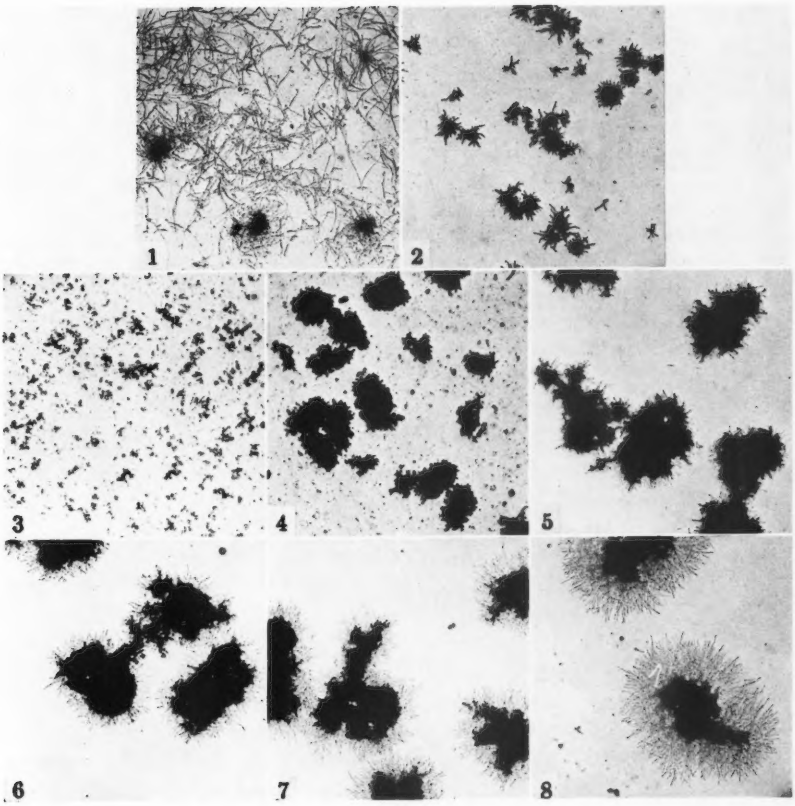

FIG. 1. Filamentous type.
FIG. 2. Retarded type.
FIGS. 3–8. Developmental stages of standard pellet-type.
FIG. 3. Immature stage (14 hr.).
FIG. 4. Early prestandard stage (16 hr.).
FIG. 5. Middle prestandard stage (18 hr.).
FIG. 6. Late prestandard stage (20 hr.).
FIG. 7. Standard stage (22 hr.).
FIG. 8. Poststandard stage (25 hr.).

Of the three factors studied, pH appeared to have the most pronounced effect on the morphology of the inoculum. The useful range was quite narrow and was common to all samples of beet molasses tested. Increase in pH from 6.0 to 7.0 decreased the rate of pellet development (Tables I and III) but this could be counteracted by increasing the spore level (Table III). The extremes of pH, 5.0 and 8.0, were unsatisfactory for production of the desired seed, while at pH 4.5 or lower the spores did not germinate. Ferrocyanide concentration was also important in controlling type of growth and rate of development of the inoculum. Too low a level resulted in loss of the characteristic pathological morphology while too high a level retarded growth excessively. The latter condition, however, could be counteracted by increasing the spore concentration (Table II).

Tests made with Chatham 1950, 1951, and 1953 molasses showed that each sample required individual treatment to produce a "standard" seed. In addition, tests made with samples from different lots of Chatham 1951 molasses showed that the variation between lots was sufficient to require individual treatment. However, with a well-mixed batch of molasses, the "standard" seed type was obtained weekly for almost one year without changing the conditions of treatment.

Fermentation

It has been shown in the previous section that type and rate of mold growth are controllable. This, however, does not necessarily imply suitable fermentative properties, i.e. reproducibility and high yield, in the inoculum developed. The "standard" inoculum was therefore tested in the submerged fermentation of ferrocyanide-treated beet molasses.

Methods

The scale of preparation of the standard inoculum was increased for fermentation studies. Comparable aeration was obtained in the larger incubation vessels by adjusting the shaker speed and liquid volume to give the same rate of oxidation (2) as that found in the smaller vessels used in the "standard" inoculum work. The seed cultures were prepared as follows: well-mixed Chatham 1951 molasses was diluted with tap water, adjusted to pH 5.4 with hydrochloric acid, dispensed in 1.5 liter aliquots to 6-liter Erlenmeyer flasks and sterilized. During sterilization (25 min. at 15 p.s.i.g.) the pH rose to 6.0. Immediately after the mash was removed from the autoclave, 0.5 gm. potassium ferrocyanide trihydrate per liter was added. When the mash had cooled to room temperature, 0.5 gm. dibasic potassium phosphate trihydrate per liter was added. Each flask was inoculated with 10^9 spores and incubated on the shaker at 28° C. The estimated pellet count of pooled cultures varied from 5 to 8 $\times$ 10^3 pellets per ml.

The fermentation mash was prepared in the same way as the seed mash except that the pH was adjusted to 6.0, and the poststerilization pH was adjusted to 7.0. The fermentations were carried out in 2.5-liter glass

fermenters similar to those described by Martin and Waters (6). The mash temperature was controlled at 31° C. The fermenters were inoculated with 200×10^3 pellets per liter of mash. The techniques of aeration, oxygenation, and foam control as well as the analytical methods have been described previously (6). Extreme care was taken to eliminate possible sources of variation in each step of the process.

Results

The acid yields obtained from the first 10 runs including a total of 23 fermentations are listed in Table IV. The average yield of anhydrous citric acid was 8.9% (68.5% conversion of available sugar). The reproducibility of the fermentation was shown by the low standard deviations of the total acid and citric acid yields, which were 3.6% and 4.5% respectively of the mean yields. The acid ratio (citric acid to total acid as citric) varied between 75 and 85%. These fermentations were characterized by the following features: uniform mold pellets about 1–2 mm. in diameter, free movement of the pellets across the aeration disc, finely dispersed aeration in the form of small bubbles, and fairly vigorous agitation of the culture.

Suddenly and without apparent cause, a breakdown of the fermentation process occurred. The results in Table V, which lists the acid yields during the breakdown period, indicate that conversion of available sugar was decreased by 50% compared with the earlier fermentations (Table IV). In addition, the reproducibility was poor (standard deviation, 28.5% of the average yield). Most of the poor fermentations were characterized by excessive mold growth which produced pellets 3–4 mm. in diameter. In

TABLE IV

ACID YIELDS OF FIRST TEN RUNS*

Run No.	Average yield at 116 hr.	
	Total acid as citric, %	Anhydrous citric acid, %
1 (3)†	10.3	8.4
2 (3)	11.3	8.5
3 (2)	10.9	8.9
4 (3)	11.3	8.6
5 (2)	10.9	9.1
6 (2)	11.7	9.2
7 (2)	10.7	8.8
8 (2)	11.2	8.9
9 (2)	11.2	9.6
10 (2)	11.1	9.5
Mean	11.1	8.9
Standard deviation	0.4	0.4
% Conversion of available sugar	85.4	68.5

* *Acid yields in this and succeeding tables not corrected for evaporation (about 5%).*
† *Number of fermentations.*

TABLE V

ACID YIELDS DURING BREAKDOWN PERIOD

Run No.	% Total acid as citric (average) at maximum titer†
11 (2)*	4.8
12 (2)	7.7
13 (2)	7.4
14 (2)	4.5
15 (2)	7.0
16 (5)	3.7
17 (4)	4.0
18 (2)	5.8
Mean	5.6
Standard deviation	1.6
% Conversion of available sugar	43.1

* *Number of fermentations.*

† *Many fermentations were discontinued before 116 hr. because of aeration difficulties (see text).*

addition, the pellets usually packed on the aeration disc early in the fermentation (from 24–48 hr.). In a few fermentations, the pellet size was normal (1–2 mm.) but the mash became viscous and sirupy presumably because of production of polysaccharide material. Packing of the pellets on the aeration disc and increased viscosity both reduced effective aeration.

The examination of a number of possible causes for the breakdown of the fermentation such as air supply, antifoam, chemical reagents, contamination, water supply, sterilization, fermenters and accessories, and molasses failed to reveal the source of the trouble. Mutation of the organism appeared unlikely because the breakdown occurred abruptly while the stock third-transfers (see inoculum methods), which had previously given good results, were in use. This conclusion was confirmed when cultures brought up from different soil stocks on fresh media gave the same poor results.

However, marked differences in acid yield were obtained with inocula prepared from sporulation cultures at various stages of transfer (Table VI).

TABLE VI

ACID YIELDS OBTAINED FROM SUCCESSIVE TRANSFERS ON SYNTHETIC SPORULATION MEDIUM

Run No.	% Total acid as citric at maximum titer			
	Transfer No.			
	1	2	3	4
19	10.3 (4)*		4.1 (3)	5.2 (4)
20	10.4 (2)	9.3 (2)	11.1 (2)	5.6 (2)
21	10.7 (2)			5.5 (2)
Mean	10.5	9.3	7.6	5.4

* *Number of fermentations.*

First-transfers from soil gave good fermentations while other transfers, in general, gave lower yields. The average yield decreased with successive transfer from soil. A random effect was also apparent since third- and fourth-transfers occasionally produced a good fermentation.

Since acid yields were normal when first-transfers were used, it was thought that a nutritionally-richer sporulation medium would be desirable (first-transfers were considered nutritionally-rich because soil is spread on the surface of the medium). Accordingly, a medium of the following composition (9) was tested:

Molasses	300 gm.
KH_2PO_4	0.50 gm.
Agar	20 gm.
Distilled water to one liter	
Poststerilization pH 6.0	

Seed cultures prepared from spores grown on this medium were similar in appearance to those prepared on the synthetic medium, and reacted in a similar manner to the alteration of variables.

The results of 12 fermentations using the molasses sporulation medium are presented in Table VII. The average yield of anhydrous citric acid was 8.6%, which corresponds to 66.2% conversion of available sugar. The variability of yields between runs was comparatively small since the standard deviation was only 2.3% of the average citric acid yield. Statistical comparison of the data in Table IV and VII failed to indicate a significant difference in yields. The contention that a nutritionally-rich medium was desirable was further supported when second- and third-transfers on molasses agar gave good fermentations and comparable transfers on the synthetic medium, tested simultaneously, were unsatisfactory.

TABLE VII

ACID YIELDS OBTAINED WITH MOLASSES–AGAR SPORULATION MEDIUM

Run No.	Fermentation time, hr.	Average yield	
		Total acid as citric, %	Anhydrous citric acid, %
22 (2)*	94	10.2	8.4
23 (4)	100	10.7	8.9
24 (2)	116	11.1	8.6
25 (2)	115	11.2	8.6
26 (2)†	116	11.2	8.4
Mean yield		10.9	8.6
Standard deviation		0.4	0.2
% Conversion of available sugar		83.8	66.2

* *Number of fermentations.*

† *After this run the supply of Chatham 1951 molasses was depleted. Subsequently, in 23 runs involving about 300 fermentations, consistently high yields were obtained with Chatham 1953 molasses. These results are not reported since fermentation conditions are not strictly comparable.*

Discussion

A "standard" predictable seed-type, uniform in morphological development, can be produced under carefully controlled conditions. The effects of pH, ferrocyanide, and spore levels were found to be related and a change in one of these factors could be counteracted by a change in one of the other two. Although the effect of varying these factors was similar for all samples of molasses tested, different levels were found to be optimum for molasses of each crop year as well as for different lots within the same crop year.

The inhibitory effect of ferrocyanide on mold growth is generally attributed to reduction in trace metal concentration. However, an increase in spore inoculum would not be expected to overcome a trace metal deficiency. Hence the increase in mold growth with increase in spore level observed in this investigation suggests that the ferrocyanide may also exert a toxic effect.

Although the immediate cause of the fermentation breakdown which occurred during this investigation was not determined, the results suggest that serial transfer on the synthetic medium made the culture more sensitive to variations inherent in fermentation technique (Table VI). Serial transfer from the soil culture to eliminate carry-over of trace elements, which was reported necessary with a synthetic fermentation medium (7), was not required in this study using beet molasses. Further, a nutritionally-rich medium gave sporulation cultures with stable fermentation properties even after serial transfer.

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STUDIES ON THE PROPAGATION OF INFLUENZA AND MUMPS VIRUSES IN TISSUE CULTURE WITH CHEMICALLY-DEFINED MEDIA[1]

By M. M. Burr, M. E. Campbell, J. F. Morgan, and F. P. Nagler

Abstract

Optimal conditions have been established for the propagation of influenza virus (PR_8 strain) in various chick embryonic tissues cultivated in synthetic medium 199. The propagation of several other influenza strains, both standard laboratory and freshly-isolated, and of mumps virus, has also been studied. Comparative investigations with virus-infected tissues cultivated in medium 199 and in simple inorganic salt solutions have shown that the extent of virus propagation is more dependent on the intracellular material present in the tissues than on the composition of the extracellular culture medium. It has also been shown that virus propagation occurs equally well in healthy, surviving cells, in actively-growing cells, and in cells depleted of nutrients and undergoing degeneration.

Introduction

The demonstration that poliomyelitis virus can be propagated in tissues cultivated in a synthetic medium (8, 19) has made it possible to study the virus–host cell relationship under closely-controlled conditions. The composition of the synthetic medium may be varied at will and the effect of such variations on the extent of virus propagation measured. From such studies, the nutritional factors required for virus propagation may eventually be determined.

Influenza virus has been propagated in chick embryonic tissues cultivated in inorganic salt solutions and in natural media (1, 6, 9), and in deembryonated eggs (3), but the effect of chemically-defined tissue culture media has not as yet been studied in this virus–host cell system. In the experiments to be reported, the propagation of several strains of influenza virus and of mumps virus has been studied in a variety of chick embryonic tissues cultivated in synthetic tissue culture medium 199 (16). It has been the purpose of this investigation to determine the optimal conditions for virus production and to evaluate the contribution of the extracellular culture medium to virus propagation within the host cells.

Materials and Methods

Virus Methods

In the majority of the experiments, the egg-adapted PR_8 strain of influenza *A* virus was used. In addition to the PR_8 strain of influenza *A*, standard and recently-isolated strains of influenza *A*-prime, a recently-isolated strain of influenza *B*, and the standard Enders strain of mumps virus were similarly

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Contribution from the Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Ontario.

studied. Seed virus for the experimental cultures was obtained from a freeze-dried pool of infected allantoic fluid with a 50% egg-infectivity titer (EID_{50}) of approximately $10^{-7.5}$. To determine the amount of the viral inoculum in each experiment, a titration of the seed virus was performed in eggs simultaneously with the inoculation of the tissue cultures.

The amount of virus produced in the tissue cultures was determined by removing at selected intervals 0.1 ml. amounts of supernatant fluid. Serial 10-fold dilutions of this material were inoculated into the allantoic sacs of 10- to 11-day-old embryonated eggs which were then incubated for 72 hr. at 37.5° C. In tests with mumps virus, 7- to 9-day-old embryos were employed and the incubation period was five days at 37.5° C. The allantoic fluids were removed and tested for the presence of virus by the hemagglutination method. From the results of these tests, the EID_{50}'s were calculated by the method of Reed and Muench (17).

In all experiments, the seed virus was obtained from large pools of freeze-dried infected allantoic fluids, and no attempt was made to adapt any of the virus strains to tissue culture by serial passages.

Tissue Culture Methods

In the majority of the experiments, the conventional Maitland flask or suspended cell technique (15) was employed. The tissue used, unless otherwise specified, consisted of pools of chorioallantoic membranes from 11-day-old embryonated eggs. Appropriate amounts of tissue were minced to a fine brei with sharp scissors, centrifuged at 1000 r.p.m. for one minute, and the supernatant fluid removed. The minced fragments were resuspended in three volumes of balanced salt solution, recentrifuged, and finally diluted 1 : 1 with salt solution. The calculated amount of tissue suspension, usually 100 mgm. wet weight, was transferred with a graduated capillary pipette to the bottom of 25-ml. pyrex Erlenmeyer flasks, 3.0 ml. of balanced salt solution added, and the cultures incubated overnight. On the following day, the supernatant fluid was removed and replaced by 2.7 ml. of medium 199. The appropriate dilution of virus in 0.3 ml. of 199 was added, the flasks tightly stoppered and incubated at 37.5° C. without further change of fluid for the duration of the test.

In certain experiments, various chick embryonic tissues were cultivated by the roller tube technique (11), either directly on the glass wall of the tubes or in plasma clots. In order to deplete the stores of intracellular nutrients, certain of these cultures were maintained in balanced salt solution for prolonged periods of time before addition of synthetic media and inoculation with virus. Details of the depletion method will be described more fully in the experimental results.

In some of the later experiments, cultures were prepared from three types of mouse ascitic tumor cells[2]: the Ehrlich, the TA_3, and the Gardner lymphosarcoma. These tumors were carried in the appropriate mouse strains by

[2] *Obtained through the courtesy of Dr. G. A. LePage, McArdle Memorial Laboratory, University of Wisconsin, Madison, Wisconsin.*

serial passages. Ascitic fluid was withdrawn from the peritoneal cavity by a syringe. This material was centrifuged and the sedimented cells washed in three volumes of distilled water to hemolyze any erythrocytes present. Cultures were prepared by transferring approximately twenty million cells, estimated by direct hemocytometer counts, to Carrel D 3.5 or T-15 culture flasks and adding 2.0 ml. of medium 199. After overnight incubation, a large proportion of the cells were found to be adherent to the glass surface and inoculation with virus could be carried out.

Cultures for virus studies were also prepared from the *L* strain of mouse cells[3] (18). This strain, which is propagated by cell suspension techniques (5), was cultivated under conditions similar to those employed with the ascitic tumor cells.

All experiments were carried out at least in duplicate and all cultures were randomized before their initial treatment. The egg infectivity titers reported are averages of several determinations or represent values obtained from single determinations on pooled samples from replicate cultures. In the majority of the experiments in which chorioallantoic membranes were employed, 100 mgm. wet weight of tissue, corresponding to 3.7 mgm. dry weight, was used. This simple measurement was found to yield reproducible cultures and, consequently, nitrogen determinations on the tissue fragments, as carried out by Womack and Kass (21), were considered unnecessary.

Tissue Culture Media

In early experiments, synthetic medium 199 (16) served as the culture fluid and Earle's balanced salt solution (4) was employed as the inorganic control solution. In later experiments, medium 199 prepared in Hanks' salt solution (12) was found to have certain advantages and this modified medium, M150, was routinely employed. Comparative experiments showed that medium M150 supported virus propagation to the same extent as medium 199 and also maintained cell survival for the same period. The reduced level of sodium bicarbonate (1.4 gm. per liter in M150 as compared with 2.2 gm. per liter in 199) gave a pH of 7.4 and made it unnecessary to control the excess alkalinity by gas mixtures rich in carbon dioxide.

Penicillin and streptomycin were incorporated in the media at levels of 100 units per ml. and 100 μgm. per ml., respectively, during the preliminary incubation period and at half these levels during the subsequent experimental period. Extensive investigations showed that penicillin retained its full activity in M150 when stored for eight days in the refrigerator and that approximately 25% of the activity remained after eight days in the incubator. The rate of penicillin inactivation was not altered by the presence of membrane tissue fragments, and it was assumed, therefore, that the antibiotic levels employed were adequate to control chance bacterial contamination.

[3] *Obtained through the courtesy of Dr. W. R. Earle, National Cancer Institute, Bethesda, Maryland.*

Experimental

Effect of Age of Embryos

Routine propagation of the egg-adapted PR_8 strain is carried out in 10- to 12-day-old embryos. It was considered of interest, therefore, to determine whether the age of embryo from which the chorioallantoic membranes were obtained would affect the growth pattern of the virus in suspended cell cultures in medium 199. Membranes of 9-, 11-, 13-, and 16-day-old embryos were selected in order to test materials both older and younger than those ordinarily used. The results obtained (Fig. 1) show that the amount of virus produced decreases slightly with increasing age of embryo. On this basis, the 9-day embryonic tissue appears to be the one of choice for virus production. However, membranes from 9-day embryos yielded relatively small amounts of tissue and were difficult to manipulate in the preparation of large tissue pools. Consequently, it was decided to use membranes from 11-day-old embryos in further studies.

It is of interest to note that Fulton and Armitage (9), by direct measurement of hemagglutinins present, found no difference in virus production when membranes of embryos ranging in age from 9 to 16 days were used. In the present experiments, using egg infectivity tests, a consistent difference was observed but the magnitude of the effect was not great.

Optimal Amount of Chorioallantoic Tissue

In Table I is shown the relationship between the amount of chorioallantoic tissue (expressed as milligrams wet weight per flask) and yield of virus by the

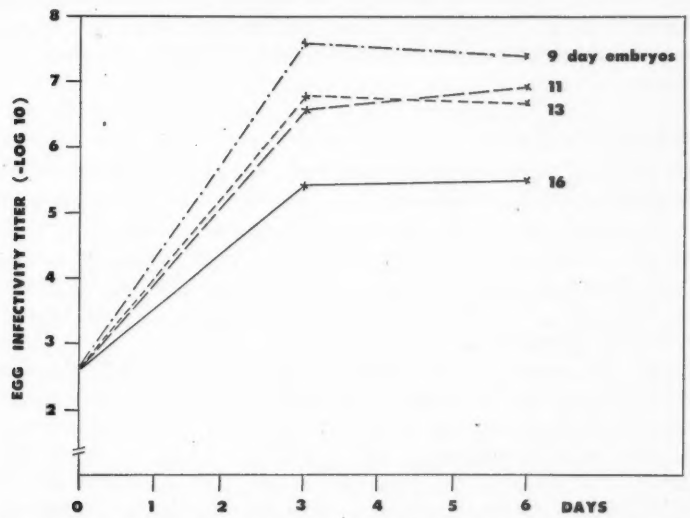

FIG. 1. Propagation of influenza virus (PR_8 strain) in cultures of chorioallantoic membrane tissue from embryos of different ages.

suspended cell technique in medium 199. It is evident that quantities of 10 and 50 mgm. give negative or variable results, and that levels of tissue from 100 to 250 mgm. yield essentially similar virus titers.

TABLE I

EFFECT OF VARYING QUANTITIES OF CHORIOALLANTOIC MEMBRANE TISSUE ON INFLUENZA VIRUS PROPAGATION (PR_8 STRAIN) IN TISSUE CULTURES

Expt. No.	Mgm. of tissue per 3 ml. of medium 199	Number of flasks	Virus inoculated ($-\log_{10}EID_{50}$)	Virus recovered ($-\log_{10}EID_{50}$) at 3 days	Virus recovered ($-\log_{10}EID_{50}$) at 6 days
A	10	2	1.6	0*, 0	0, 0
A	50	2	1.6	0, 4.0	0, 6.4
B	50	2	2.3	0, 6.0	0, 6.6
C	50	3	2.4	0, 6.5, 6.8	0, 6.3, 6.0
B	100	1	2.3	7.4	6.6
C	100	7	2.4	7.5 (Av.)	6.7 (Av.)
B	150	2	2.3	6.8, 7.6	7.1, 7.2
B	200	1	2.3	6.9	6.5
A	250	2	1.6	6.5, 7.0	6.5, 6.0

*$0 = < 10^{-2}\ EID_{50}$.

Additional experiments, in which replicate flask cultures were prepared containing 100 mgm. wet weight of chorioallantoic tissue, showed that variation in virus titers was very small. It was considered, therefore, that pooling of samples from duplicate or triplicate flasks would adequately compensate for any flask variations. These observations agree reasonably well with the recent findings of Gajdusek (10) on the reproducibility of influenza titers in suspended cell cultures.

Optimal Virus Inoculum

In the foregoing experiments, the test flasks were inoculated with sufficient virus to yield an EID_{50} of approximately $10^{-2.0}$. In order to determine the optimal amount of virus inoculum, replicate test flasks were seeded with four levels of virus, to provide EID_{50}'s of $10^{-3.6}$, $10^{-2.6}$, $10^{-1.6}$, and $10^{-0.6}$. Results of this experiment are shown in Fig. 2. Titers obtained at the $10^{-0.6}$ level are not shown in this graph because no virus propagation could be detected throughout the experiment.

While the two larger inocula produced higher virus titers in the first two days, by the third day the titer for all three levels was approximately the same and remained so up to the sixth day. It would appear, then, that under these experimental conditions, a viral inoculum of approximately $10^{-1.6}$ is necessary to initiate influenza propagation and that quantities of virus in excess of this figure do not significantly affect the final titers. Based on these results, a viral inoculum of approximately $10^{-2.0}$ was employed in all subsequent experiments.

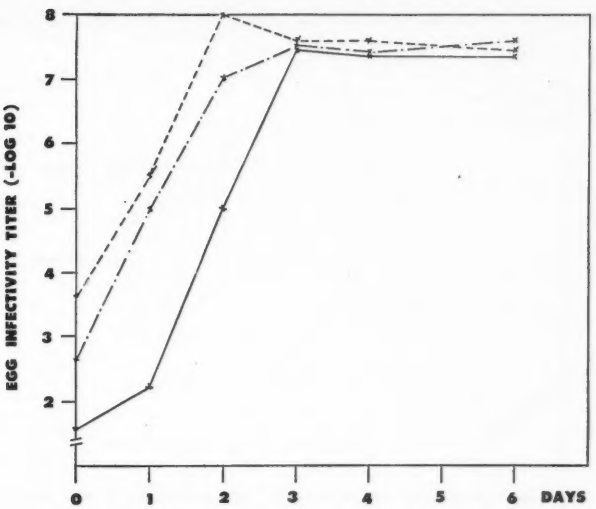

FIG. 2. Relationship between amount of virus inoculum and propagation of influenza virus (PR_8 strain) in chorioallantoic membrane cultures.

Influenza Propagation in Different Tissues

Since the experiments up to this point had been carried out entirely with chorioallantoic membrane, it was considered of interest to determine the extent of influenza propagation in other chick embryonic tissues, using the suspended cell technique in synthetic medium 199. From the results of this experiment, Fig. 3, it is apparent that chorioallantoic membrane and lung produce the highest titers, that liver supports moderate virus production, and that heart muscle is completely unsuitable.

Results with cultures of brain tissue were also negative as no consistent evidence of virus propagation was obtained. These findings with chick embryonic brain tissue are at some variance with the results of Weller and Enders (20) but are in agreement with earlier experiments of Francis and Moore (7).

Behavior of Different Virus Strains

Since these results had been obtained entirely with the PR_8 strain, the behavior of other virus strains under similar cultural conditions was next investigated, employing both standard laboratory and freshly isolated strains.

With all four strains of influenza studied, the pattern of virus propagation appeared to be essentially similar. The standard and the freshly isolated *A*-prime strains showed propagation curves that were almost identical with each other, and that were only slightly lower in titer than the PR_8 reference strain. The influenza *B* strain propagated more slowly and to lower titer, but the shape of the propagation curve was similar to that of the other influenza strains tested. Propagation of mumps virus (Enders strain) was characterized by slow development to maximum titer at six to eight days (see Fig. 6).

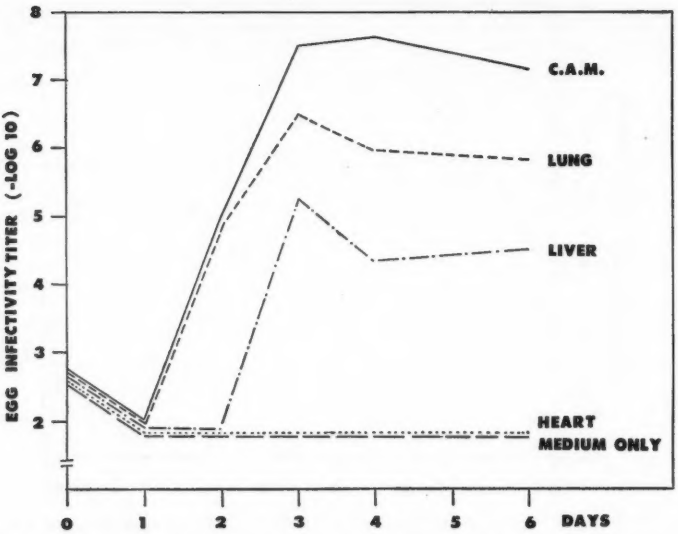

FIG. 3. Propagation of influenza virus (PR_8 strain) in various chick embryonic tissues cultivated in medium 199.

From these results, it was concluded that the egg-adapted PR_8 strain was generally representative of the behavior of influenza strains in tissue culture and that mumps virus, because of its slow propagation in tissue culture, should be included for comparative purposes in subsequent experiments.

Studies with Specific Cell Types

In addition to studies with various chick embryonic tissues, the propagation of influenza virus was also investigated in cultures of the *L* strain of mouse cells and in cultures of three different ascitic tumor cells. In no case was any evidence of virus multiplication obtained. These negative findings are in general agreement with the observations of Koprowska and Koprowski (13) that influenza virus gave inconsistent results when inoculated into mice bearing the Ehrlich ascitic tumor. Ackermann and Kartz (2) have recently shown, however, that adaptation of the cells to the virus must be carried out by simultaneous passages in mice before virus propagation can be demonstrated in these cells *in vitro*, and this possibility is being investigated.

Virus Propagation in Different Media

In order to determine the effect of composition of culture fluid upon virus propagation within the cells, comparative experiments were carried out by the suspended cell technique with chorioallantoic membrane cultures maintained in 199, in Earle's balanced salt solution, and in the Hanks–Simms' serum ultrafiltrate medium widely used for virus studies (20). The results of these experiments are presented in Figs. 4 and 5. It is evident that these three

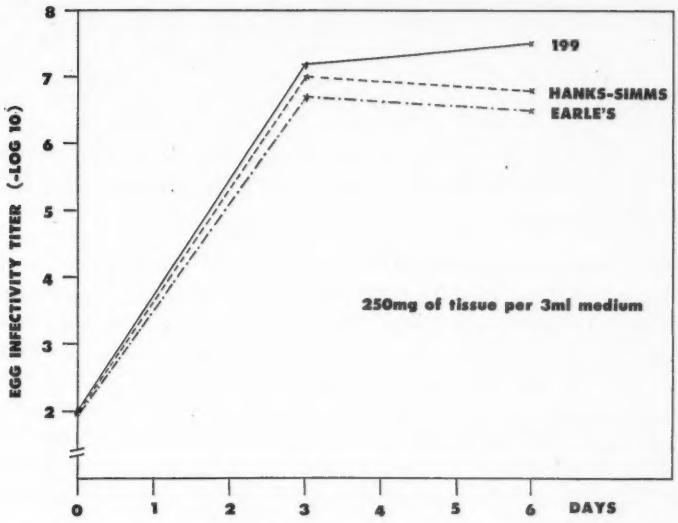

FIG. 4. Propagation curves of influenza virus (PR_8 strain) from chorioallantoic membrane tissue (250 mgm. per culture) cultivated in three different media.

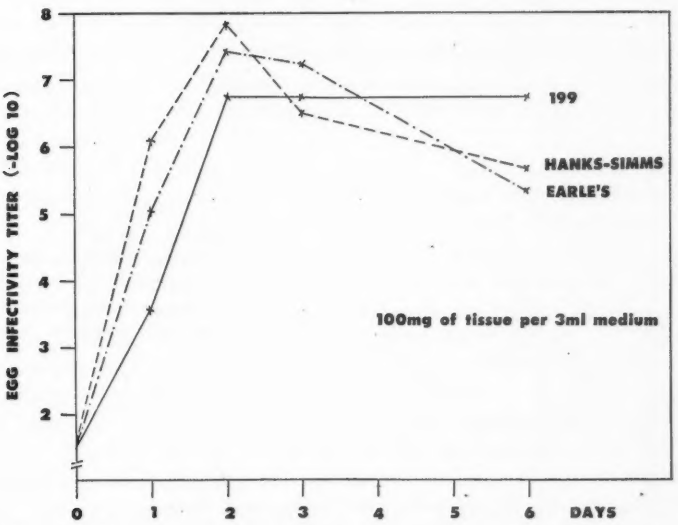

FIG. 5. Propagation curves of influenza virus (PR_8 strain) from chorioallantoic membrane tissue (100 mgm. per culture) cultivated in three different media.

media have supported virus propagation to approximately the same extent, whether or not excessive quantities of tissue were used. In both the salt solution and the serum ultrafiltrate media, there was a gradual drop in virus titer beyond the second day while in 199 the virus titer remained constant up to the sixth day. A characteristic feature of the virus propagation curve obtained with 199, in these and several other experiments, was the maintenance of the virus titer close to its maximum for at least one week once this high level had been reached.

Effect of Cell Depletion Techniques

The observation that equal amounts of virus were produced in chorioallantoic tissue whether the culture medium consisted of complete 199 or its inorganic salt components suggested that virus propagation proceeded independently of the extracellular fluid. It became of interest, then, to attempt to deplete the stores of intracellular nutrients by various culture techniques and to determine what effect such depletion might have on subsequent virus propagation. In these studies, the PR_8 strain of influenza virus and the Enders strain of mumps virus were used, together with M150 and Hanks' salt solution.

Depletion of intracellular nutrients was first attempted by incubation of the suspended chorioallantoic fragments in Hanks' solution for six days. After this period, the supernatant fluid was removed and was replaced either by M150 or by fresh Hanks' solution, and the virus inoculated. Even after this treatment of the tissue, as much virus was produced by the cultures in Hanks' solution as in M150 and the shape of the propagation curve was normal.

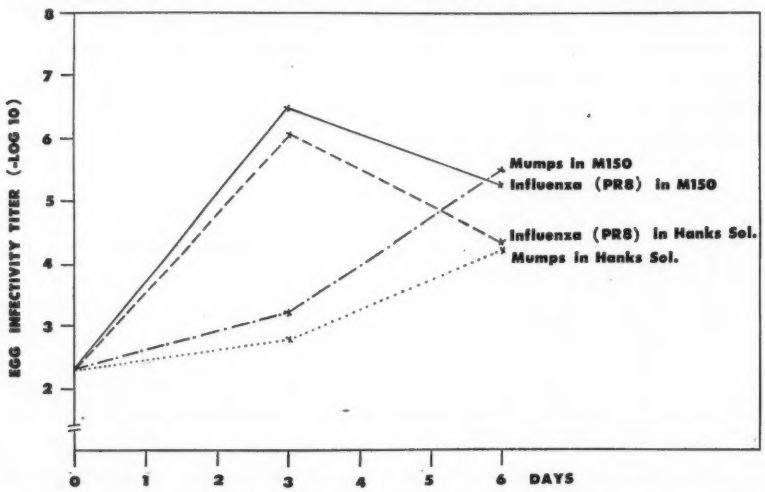

FIG. 6. Propagation of influenza and mumps viruses in chorioallantoic membrane tissues cultivated in M150 and Hanks' salt solution, after depletion of intracellular nutrients.

Since this type of cell depletion was ineffective, actively-growing cultures of chorioallantoic membrane were prepared in roller tubes, with and without plasma clots. The tissue fragments were cultivated in Hanks' solution for six days and during this period put out extensive sheets of cells which then began to degenerate slightly. At this point, the supernatant fluids were removed, either M150 or fresh Hanks' solution added, and the virus inoculated. The results of a typical experiment, with the tissues cultivated directly on the glass, are shown in Fig. 6.

With the PR_8 strain, slightly higher yields of virus were obtained in M150 than in Hanks' solution but the difference was of doubtful significance. The peak of virus production occurred at the third day, as observed previously, and there was some drop in titer by the fifth day. With mumps virus there was also a slightly higher titer in M150 than in Hanks' solution, but the propagation curve was essentially normal. In repeated experiments, differences were not significant.

From these results, it must be concluded that cultivation of chorioallantoic membrane in a simple inorganic salt solution until starvation effects were noticeable did not alter significantly the ability of the tissue cells to support the subsequent propagation of influenza or mumps virus.

Discussion

The present experiments have shown that the PR_8 strain of influenza *A* virus propagates readily in cultures of chorioallantoic membrane maintained in synthetic medium 199 or in balanced salt solutions. The propagation curves obtained coincide closely both in titer and rate of virus formation with the propagation curves obtained in embryonated eggs. Similar results were obtained with other influenza strains, both established laboratory and freshly-isolated types.

It has been shown that virus propagation in tissue cultures occurs mainly at the expense of intracellular material and appears to be independent of the nature of the extracellular tissue culture medium. This was shown by repeated experiments in which comparable titers were obtained from virus-infected tissues cultivated in medium 199 and in Hanks' balanced salt solution. The propagation of influenza virus to high titer in tissues cultivated in simple inorganic solutions had previously been demonstrated (1, 9, 14, 20), but no detailed comparison employing complex synthetic tissue culture media appears to have been made of this effect.

One distinctive feature of the influenza propagation curve obtained from tissues cultivated in synthetic medium 199 is the maintenance of the titer at its maximum value for extended periods of time, usually from five to seven days. This is in marked contrast to the decline in titer beyond the third day observed when the tissues are maintained in simple salt solutions. Such a rapid drop in titer under comparable conditions has been noted previously (14).

Extensive depletion of intracellular nutrients by cultivation of the tissues for prolonged periods in simple salt solutions before inoculation with virus did

not appear to diminish significantly the ability of the cells to support the subsequent propagation of either influenza or mumps viruses. These depletion studies were carried out with both suspended tissue fragments and with actively-growing tissues for as long as six days, a period that had been shown previously (16) to be the maximum time interval before rapid cell degeneration occurred. On the basis of these studies, it would appear likely that virus formation is occurring at the expense of large-molecular materials firmly bound to the cell structure and not through the utilization of small-molecular labile materials.

Under the present experimental conditions, influenza virus appeared to propagate equally well in healthy, surviving cells, in actively-growing cells, or in cells depleted of intracellular nutrients and undergoing degeneration. This observation raises the question as to whether living cells, as such, are actually required for influenza propagation in tissue cultures. Further experiments are planned to investigate this possibility and to determine whether the virus titers obtained from degenerating cells might have been caused by a sudden liberation of virus into the medium as cell death and lysis occurred.

Acknowledgments

The assistance of Miss S. A. Fisher and Mr. L. F. Guerin is gratefully acknowledged.

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STAGES IN THE DEVELOPMENT OF TWO INSECT VIRUSES[1]

By F. T. Bird and Mary M. Whalen[2]

Abstract

Stages in the development of viruses of two Hymenopterous insects, *Diprion hercyniae* (Htg.) and *Neodiprion americanus banksianae* Roh., are shown in electron micrographs of thin sections. Following swelling of the nuclei and nucleoli and coagulation of the chromatin, rod-shaped virus particles appear chiefly on the exposed surfaces of the chromatin. The chromatin of *D. hercyniae* often coagulates into separate lumps that suggest small polyhedra. These first produce rod-shaped particles on their exposed surfaces and later transform into recognizable polyhedra. The chromatin of *N. a. banksianae* is more uniformly dispersed, and polyhedra arise as thickenings within the chromatin. In the latter insect, virus particles are frequently observed within the developing polyhedra, suggesting that polyhedra are formed by the accumulation of protein around groups of virus particles. Both polyhedra and free virus particles are larger and denser in the nuclear sap than in the chromatin. Their formation continues in remnants of chromatin in nuclei otherwise filled with large polyhedra. An incidental observation is that the nuclear membranes of some nuclei are pitted with small holes.

Introduction

From a study of thin sections and of material extracted from infected cells of *Diprion hercyniae* (Htg.) (Hymenoptera), Bird (6) concluded that there are two stages in the multiplication of an insect virus: one in the free state and one in "growing" polyhedra. Hughes (11) found that rod-shaped virus particles appear in large numbers in the nuclei of infected cells of *Colias philodice eurytheme* Bdvl. (Lepidoptera), and suggested that their formation may be related in some way to "chromatin remains". According to Hughes, polyhedra are formed by the deposition of protein around and between bundles of two or more rods. Smith and Xeros (14) believe that the chromatin mass of a virus-infected nucleus of *Bombyx mori* L. (Lepidoptera) has a fibrillar structure and that these fibrils thicken to the normal diameter of the virus rod, polyhedra being formed by the accumulation of protein around virus rods.

Studies of the development of the virus by means of thin sections of the nuclei of *D. hercyniae* were continued, and virus development in another Hymenopteron, *Neodiprion americanus banksianae* Roh., was investigated. Stained sections of *D. hercyniae* were re-examined with the light microscope. In both insects polyhedra are formed in the nuclei of the digestive cells of the mid-gut epithelium only, and in this respect differ from polyhedroses of Lepidoptera (8).

Methods

Mid-gut epithelia of healthy and diseased *D. hercyniae* larvae were fixed in Bouin's fluid, stained with Heidenhain's iron haematoxylin, sectioned, and

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examined with the light microscope. Thin sections were prepared of the mid-gut epithelia of (1) healthy *D. hercyniae* larvae, (2) *D. hercyniae* larvae at three-hour intervals after they were fed virus, and (3) *N. a. banksianae* larvae 72 hr. after being fed virus. The guts were removed, cut into small pieces (about 1 mm. square), and fixed for four hours in 1% osmium tetroxide buffered at pH 7.4 as described by Palade (13). After the guts were embedded in *n*-butyl methacrylate, they were sectioned with a microtome, similar to that described by Hodge, Huxley, and Spiro (10), constructed at this laboratory by G. H. Bergold. The microtome was fitted with glass knives.

The photographs were taken with an R.C.A., type E.M.U., electron microscope equipped with a wide-field pole piece.

Light Microscope Studies of Stained Sections

Healthy ("normal") nuclei and virus-infected nuclei of the digestive cells of mid-gut epithelium of *D. hercyniae* are shown in Fig. 1. Normal nuclei (nuclei *A*, *B*, *C*) contain a large number of nucleoli (*n*), and the chromatin (*c*) is in the form of a network. The first symptoms of infection are swelling of the nuclei and nucleoli and coagulation of the chromatin (nuclei *D* and *E*). Polyhedra (*p*) appear as small granules in the chromatin, as shown in Figs. 1 (nuclei *F* and *G*) and 12. The nuclei then become filled with immature polyhedra (nucleus *I*) and later with much larger polyhedra (nucleus *H*). Because mature polyhedra are more resistant to staining than are the chromatin and nucleoli, it is possible to demonstrate that polyhedron formation continues in remnants of the chromatin, and that enlarged nucleoli are present in nuclei otherwise filled with large polyhedra (nucleus *J*).

Electron Microscope Studies of Thin Sections

Healthy digestive-cell nuclei appear as in Figs. 2 and 3. The nucleoli (*n*) are easily recognized. The chromatin (*c*) comprises the lumps of dense material in the form of a network. The nuclear sap is in the form of either threads or small spherical particles, a variable condition which could result from differences in fixation or in treatment of the tissues after sectioning. The nuclei of regenerative cells of the mid-gut, which are not susceptible to the virus (7), differ from those of the digestive cells in that each nucleus contains a single, large nucleolus, and the chromatin is less diffuse (Fig. 4). An interesting incidental observation is that the nuclear membrane is pitted with small holes (Fig. 5). Bahr and Beermann (1) observed previously that nuclear membranes of salivary gland and mid-gut cells of *Chironomus* are perforated by numerous small holes.

The first symptoms of infection appear in nuclei of digestive cells of *D. hercyniae* 33 hr. after the larvae have been fed with virus. These consist of a swelling of the nuclei and nucleoli and coagulation of the chromatin (Fig. 7). A nucleus in an adjacent cell (Fig. 6) appeared healthy except for a

possible slight swelling of the nucleoli; the spherical particles in the nuclear sap were indistinguishable from those occurring in normal nuclei. Following the first symptoms of infection, many stages of virus development were observed in the same gut. After 48 hr., all stages were observed. Therefore, although the sequence of events could be determined by the preponderance of a certain stage of virus development in the sections, no further reference to actual time will be made.

Rod-shaped virus particles appear later in large numbers chiefly on the exposed surfaces of the chromatin of both *D. hercyniae* and *N. a. banksianae* (Figs. 8, 9, 10, 11, 13, and 16). It is only in or on the chromatin that virus particles are formed. Later, some of the particles become dissociated from the chromatin masses and appear in the nuclear sap, where they are thicker and denser than those in the chromatin (Fig. 9).

Polyhedron formation commences in the chromatin, as previously shown in light microscope studies of stained sections (Figs. 1 and 12). The chromatin of *D. hercyniae* often coagulates in such a manner that separate lumps suggest small polyhedra, on the outer surface of which rod-shaped particles first appear. The lumps then transform into recognizable polyhedra (Fig. 16). The chromatin of *N. a. banksianae* is more uniformly dispersed, and polyhedra arise as thickenings within the chromatin (Figs. 11 and 13). In the latter insect, virus particles are frequently observed within the growing polyhedra, but have yet to be detected within those of *D. hercyniae*. In both insects, the polyhedra are found in ever-increasing numbers in the nuclear sap, where they are larger and denser than those in the chromatin.

The chromatin of the nucleus decreases as virus particles and polyhedra are formed. Remnants of chromatin, however, are present in nuclei that contain large numbers of apparently mature polyhedra, and these remnants continue to produce virus particles (Fig. 15).

When purified polyhedra are dissolved in weak alkali, spherical particles, as well as rod-shaped particles, are found, and it has been shown (6) that the spheres are an early stage in the development of the rods. In most of the thin sections a large number of spherical particles are present in the nuclear sap and also in the chromatin. Many are probably cross sections of rods, but their prevalence in certain sections indicates that some, particularly those individually enclosed by a membrane (Fig. 14), are spherical stages in the development of rods.

The nucleoli continue to increase in size and become more diffuse following the first symptoms of virus infection (Figs. 9 and 16).

Discussion

Polyhedral bodies consist of about 95% by weight of non-infectious protein and 5% or less of infectious virus particles (2). Only the virus contains desoxyribonucleic acid (2), and amino acid analyses of polyhedral protein, virus, and membranes associated with the virus showed that each is significantly different (5). The virus and membranes are more similar to one

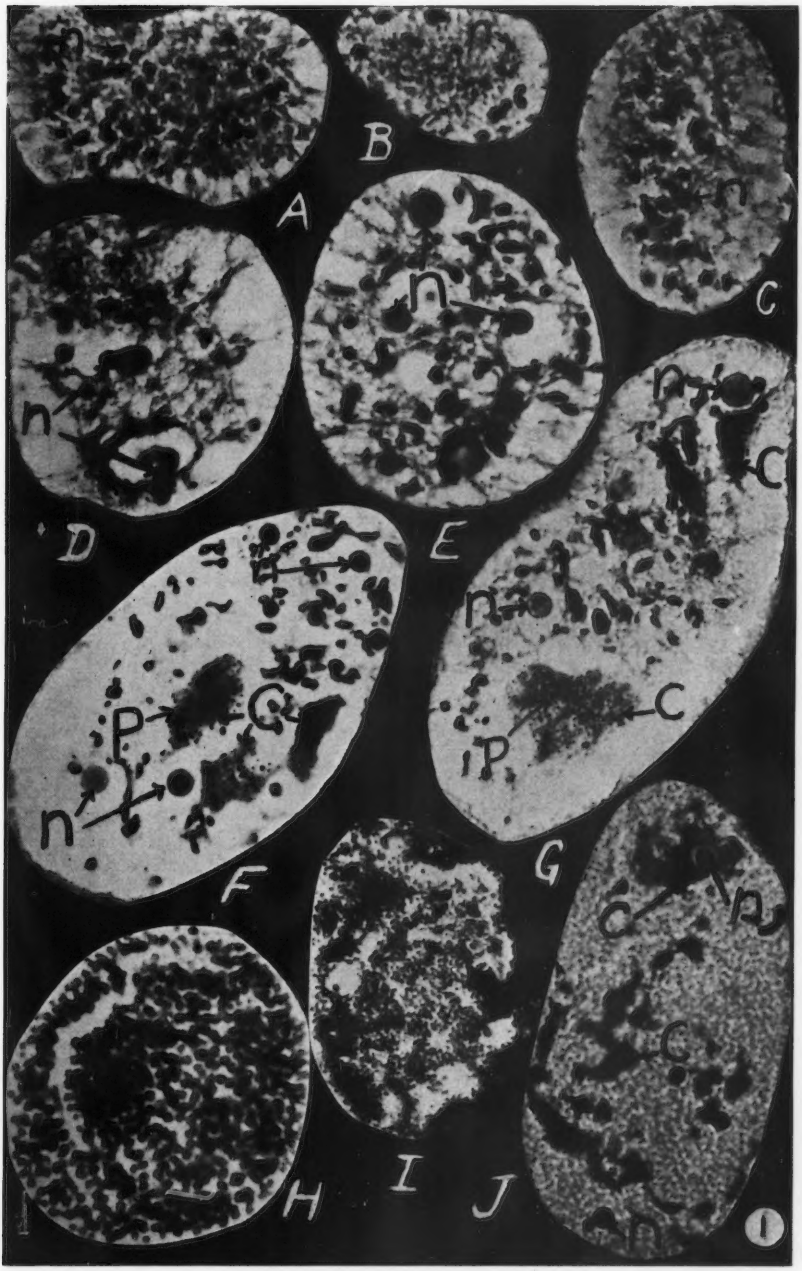
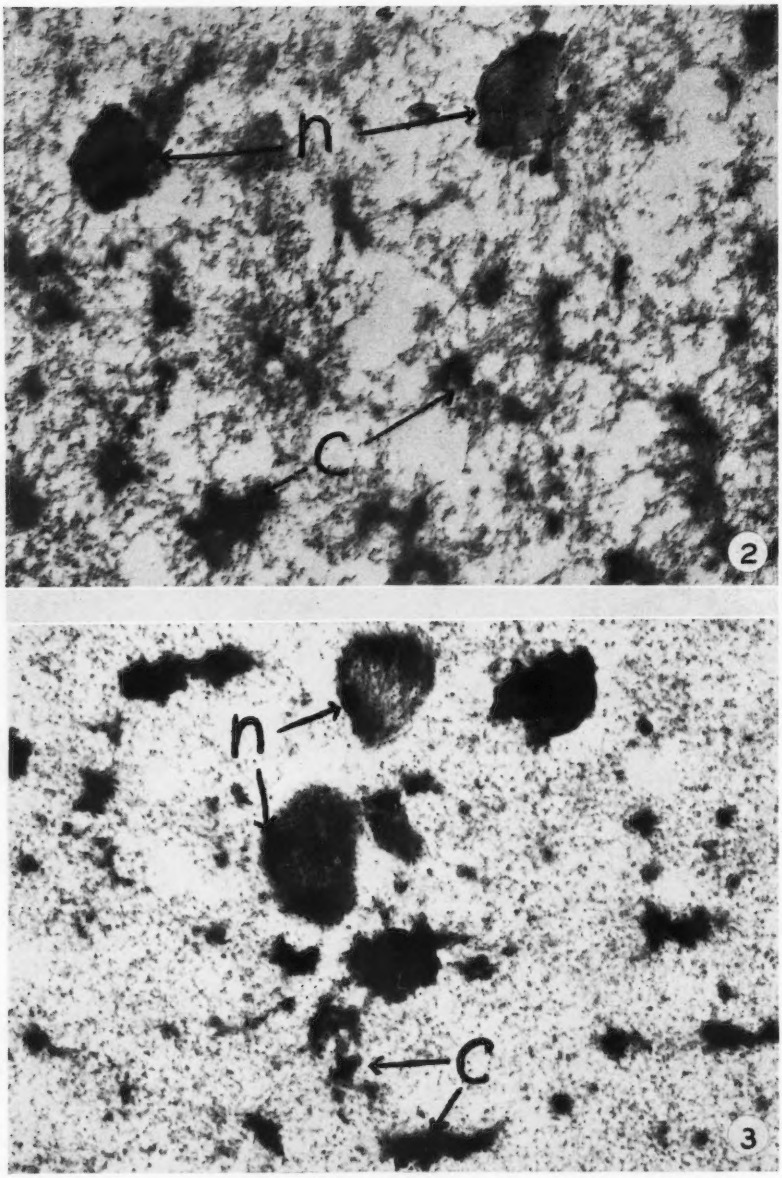
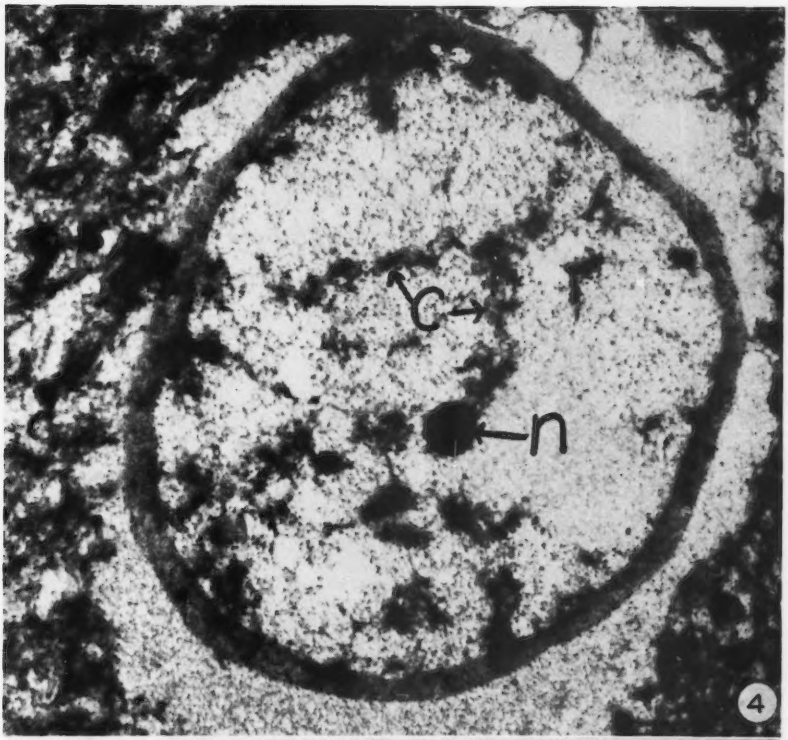


FIG. 1. Photomicrographs of healthy and virus-infected nuclei. Nuclei *A* to *C* normal. Nuclei *D* to *J* are virus-infected. Nucleolus (*n*); chromatin (*c*); polyhedra (*p*). Bouin's fixative, Heidenhain's iron haematoxylin stain. × 1150.



FIGS. 2 and 3. Healthy nuclei of *D. hercyniae*. × 10,000.

FIGS. 2–11, 13–16. Electron micrographs of thin sections of healthy and virus-infected nuclei of digestive cells, and a regenerative cell, of mid-gut epithelia of *D. hercyniae* and *N. a. banksianae*. Nucleolus (*n*); chromatin (*c*); polyhedra (*p*); rod-shaped virus particles (*v*); spherical bodies (*s*).



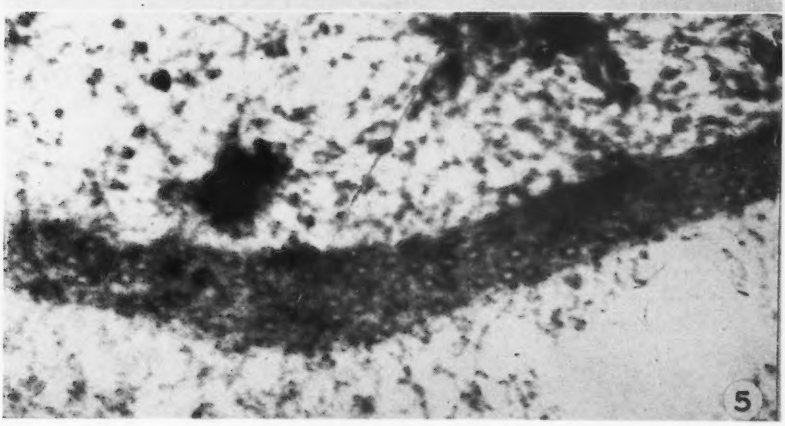
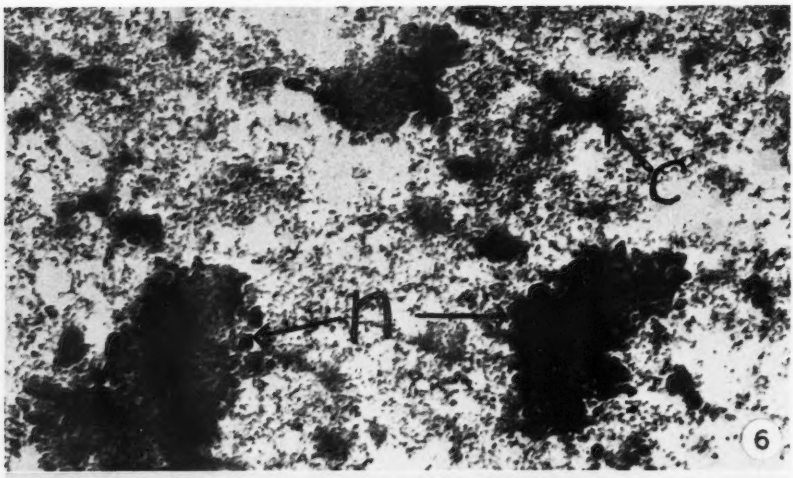


FIG. 4. Nucleus of a regenerative cell of the mid-gut epithelium of *D. hercyniae*. × 7500.

FIG. 5. Nuclear membrane of a regenerative cell showing small holes. × 25,000.



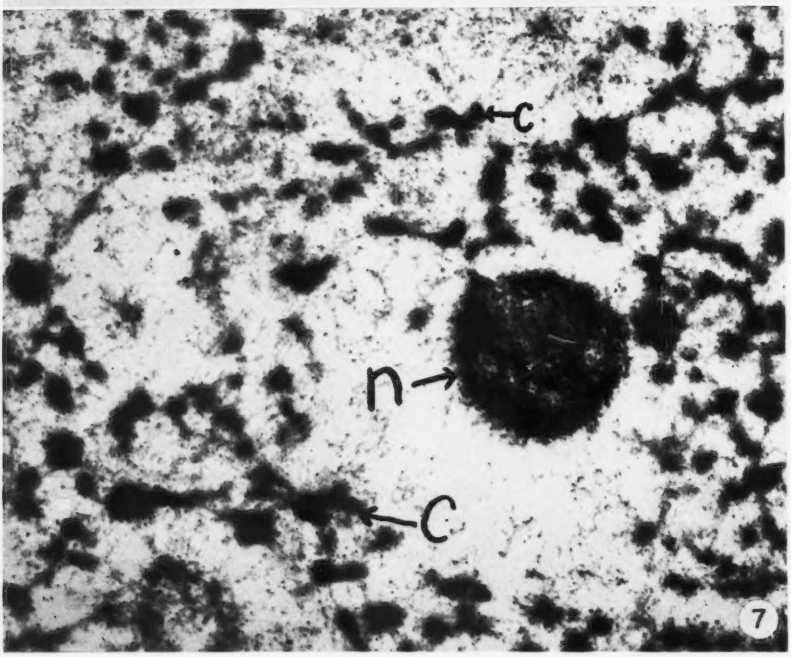


FIG. 6. Apparently healthy nucleus of a digestive cell of *D. hercyniae* observed in a virus-infected insect. × 10,000.

FIG. 7. Virus-infected nucleus of *D. hercyniae* showing early stages in chromatic coagulation. × 10,000.

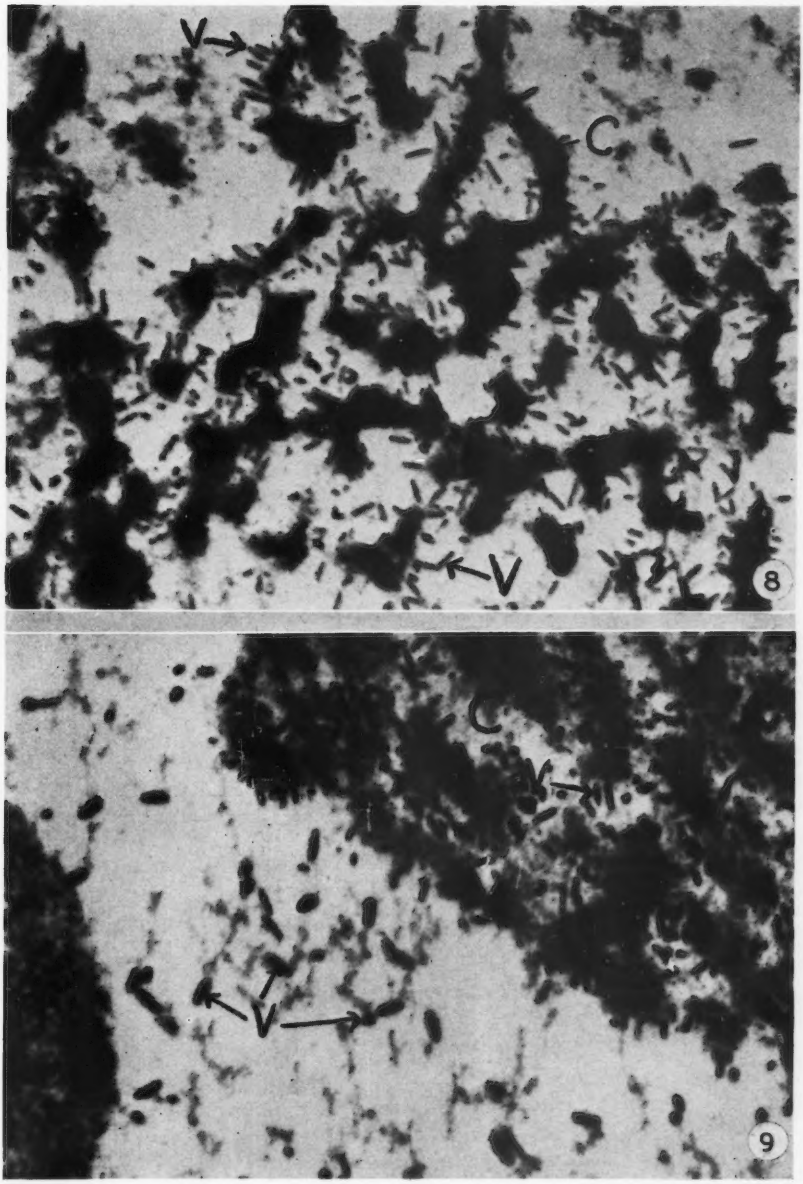


FIG. 8. Chromatin of a virus-infected nucleus of *D. hercyniae* showing rod-shaped virus particles. × 20,000.

FIG. 9. Virus-infected nucleus of *D. hercyniae* showing rod-shaped virus particles in the chromatin, part of a nucleolus, and thickened virus particles in the nuclear sap. × 20,000.

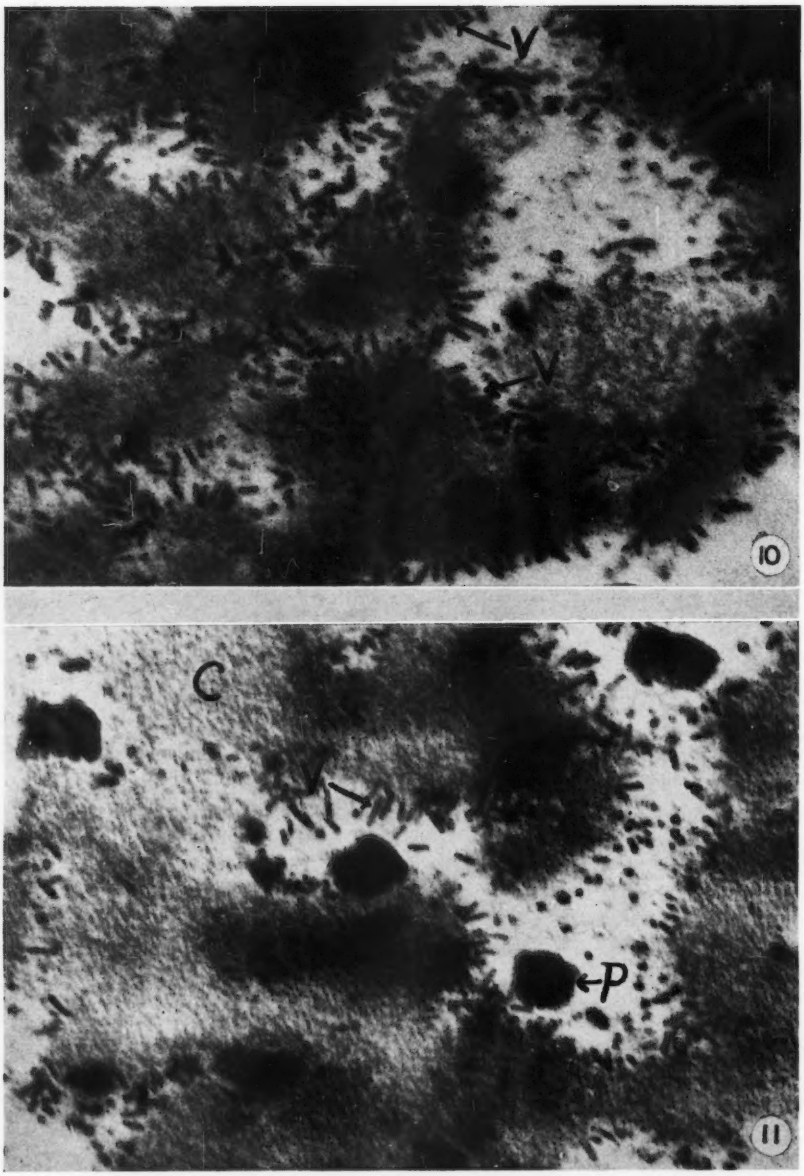


FIG. 10. Chromatin of a nucleus of *N. a. banksianae* showing rod-shaped virus particles. × 20,000.

FIG. 11. Chromatin of a nucleus of *N. a. banksianae* showing an early stage of polyhedron formation. × 20,000.

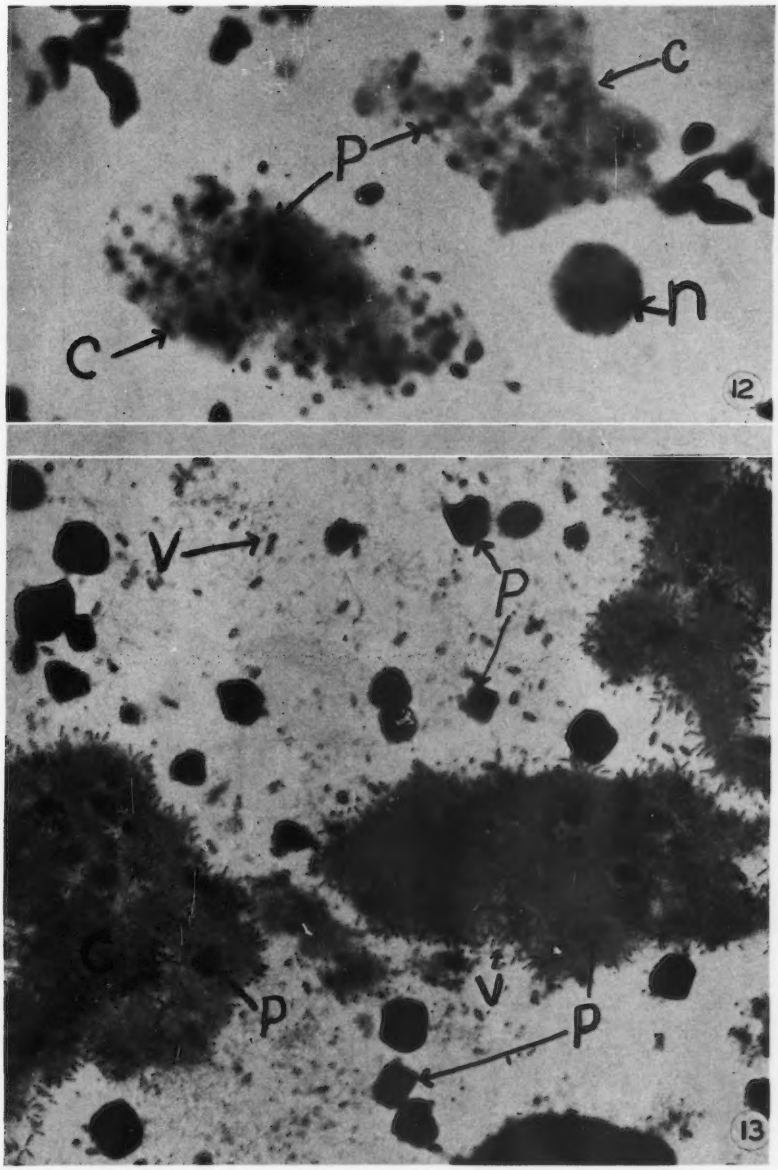
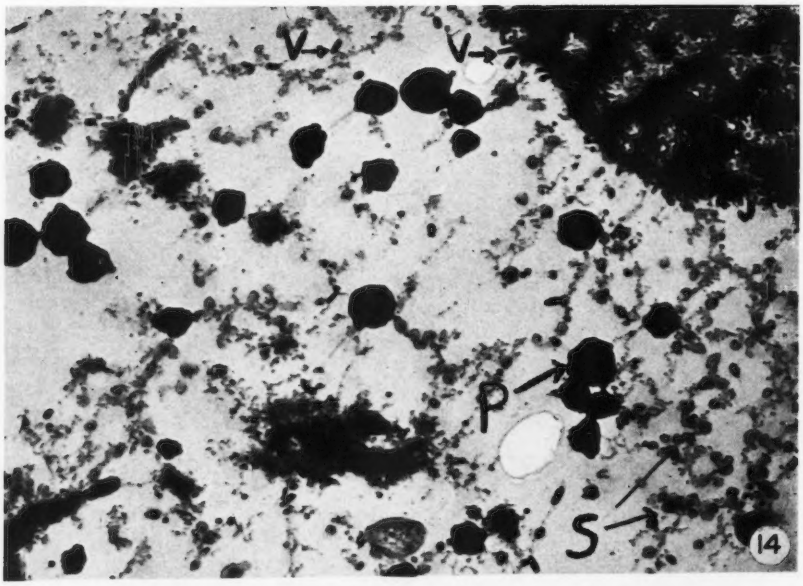


FIG. 12. Enlargement of a portion of the nucleus in Fig. 1 (*F*) showing formation of polyhedra in the chromatin. Light microscope. × 3680.

FIG. 13. Nucleus of *N. a. banksianae* showing formation of polyhedra in the chromatin. × 10,000.



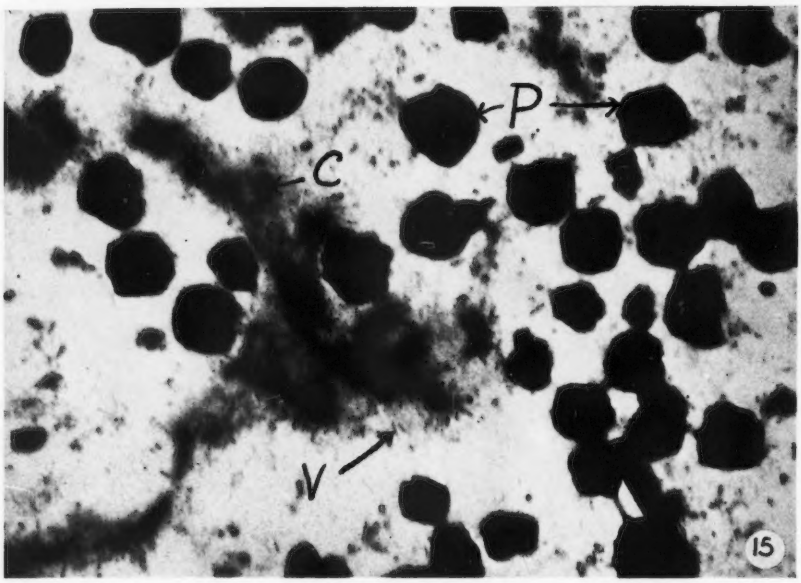


FIG. 14. Nucleus of *D. hercyniae* showing spherical bodies (*s*) in the nuclear sap. × 10,000.

FIG. 15. Nucleus of *D. hercyniae* showing greatly enlarged polyhedra and rod-shaped virus particles in chromatic remnants. × 10,000.

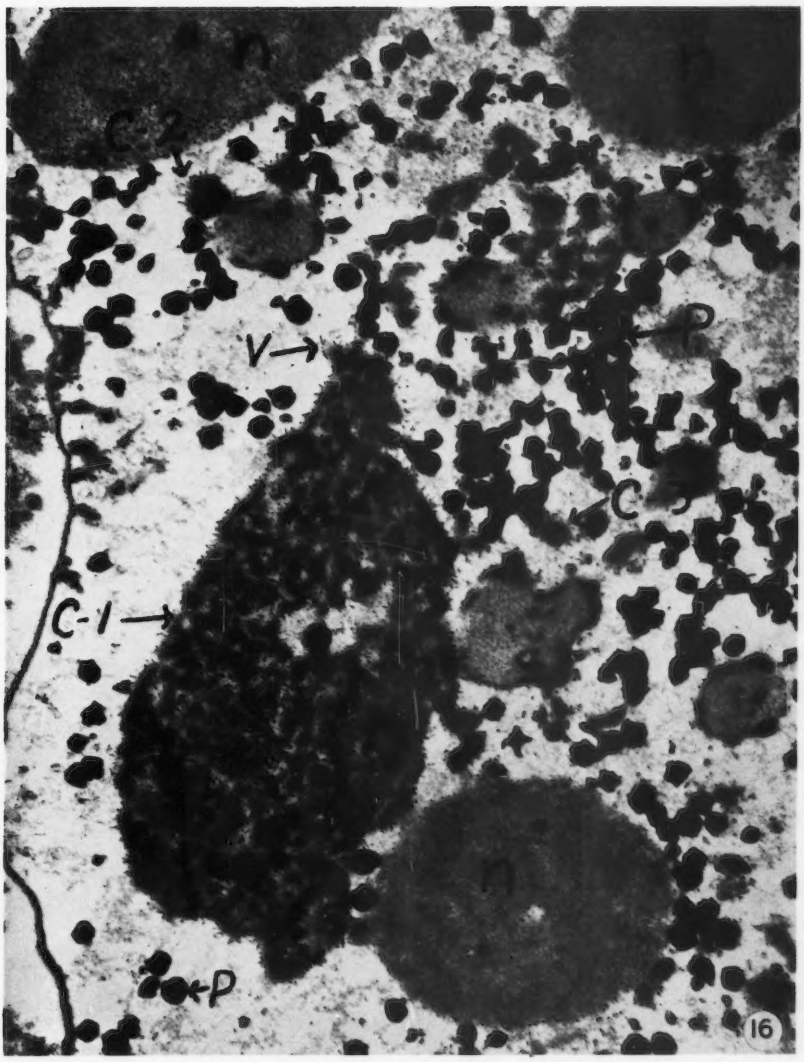


FIG. 16. Nucleus of *D. hercyniae* showing rod-shaped virus particles attached to a large mass of chromatin (*c*-1) and to small lumps of chromatin (*c*-2); an early stage in polyhedron formation (*c*-3); enlarged nucleoli (*n*); and dense polyhedra (*p*). × 5000.

another than either is to the polyhedral protein. These findings, together with the occurrence of a weak serological cross-reaction of polyhedral protein with the virus (4), suggested that the protein is a metabolic by-product of the host.

The chromatin is obviously the source of the desoxyribonucleic acid of the virus. The development of the virus takes place only in or on the chromatin, and rod-shaped virus particles, which appear chiefly on the exposed surfaces of the chromatin, resemble organisms, and not, as Smith and Xeros (14) suggest, thickenings of chromatin fibrils. The presence in the nuclear sap of spherical particles surrounded by individual membranes suggests a developmental cycle of the virus as previously described (3). Either developmental stages of the virus within the chromatin are too small, or the sections are too thick, to demonstrate stages in virus multiplication in the chromatin. Further efforts will be made to prepare thinner sections.

The source of the polyhedral protein is open to speculation. The swelling of the nucleoli, together with the body of evidence that nucleoli are concerned in protein synthesis, suggests that they may elaborate the polyhedral protein. Consistent with this idea are the results of Gratia, Brachet, and Jeener (9), who observed, in virus-infected silkworm, that the ribonucleic acid content of the nucleoli increased, as well as the desoxyribonucleic acid content of the chromatin, on the second day after larvae were infected with virus, and a further increase occurred on the fifth day.

In *N. a. banksianae*, it appears that polyhedra are formed by the accumulation of protein around groups of virus particles within the chromatin. In *D. hercyniae*, small lumps of chromatin, which first produce virus rods on their surfaces, appear to transform into polyhedra. Virus particles have not been observed within *D. hercyniae* polyhedra, probably because the latter are too dense. Further development of the polyhedra of both insects occurs, apparently, in the nuclear sap, presumably by deposition of protein. It is possible that free virus particles, which also increase in density and in size in the nuclear sap, become occluded during growth of the polyhedra as described by Hughes (11), but there is little direct evidence to support this. To the contrary, Komarek and Breindl (12) have shown that virus particles are concentrated in the center of the polyhedron, and observations during the dissolution of *D. hercyniae* polyhedra in weak alkali are in agreement.

Acknowledgments

Grateful acknowledgment is extended to Dr. G. H. Bergold for constructive discussion and criticism of the paper; to Miss Ione A. Wingfelder, who assisted in the preparation of the thin sections; and to Mr. D. C. Anderson, who assisted in photographing the stained sections and prepared all photographic prints.

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ANTIBACTERIAL ACTION OF A REACTION PRODUCT OF CYSTEINE AND IRON

I. DEVELOPMENT OF THE SUBSTANCE IN MEDIA FOR *MYCOBACTERIUM TUBERCULOSIS*[1]

By Jack Konowalchuk[2], N. A. Hinton[2], and G. B. Reed[3]

Abstract

It is shown that the growth of *Mycobacterium tuberculosis* or a rapidly growing strain of *Mycobacterium* in Dubos' medium is not influenced by the addition of low concentrations of cysteine or ferric salts, when added separately. But when cysteine and a ferric salt, both in low concentrations, are autoclaved together in the medium, the medium will not support growth of *Mycobacterium*. This inhibitory action only occurs when the cysteine–iron containing medium is at pH 6.8 or a more acid reaction. It is concluded that cysteine and ferric iron react in acid solution to produce a toxic end product.

In a study of the amino acid metabolism of *Mycobacterium tuberculosis*, Marshak (5) has shown that of 29 amino acids added to a basic synthetic medium, in which ammonium chloride was the only source of nitrogen, 13 had no influence on the growth of *M. tuberculosis*, five stimulated the growth, six retarded the growth, four retarded growth of the virulent H37 Rv but had no influence on the avirulent H37 Ra strain, one stimulated the growth of Ra but had no influence on the virulent Rv. Our results with the same 29 amino acids are in general agreement with those of Marshak.

Cystine and cysteine were not included in Marshak's list of amino acids. These substances, especially in heated solutions, have frequently been shown to be toxic for certain species of bacteria. Fildes and Richardson (2) and Woiwod (12) found cystine, when autoclaved in culture media, to be toxic for *Staphylococcus*, and Schuhardt *et al.* (9) found it, under similar circumstances, to be highly toxic for *Brucella*. Schuhardt *et al.* (10) found that the cystine per se was not toxic but that the observed toxicity resulted from breakdown products of cystine, and Schuhardt *et al.* (11) demonstrated the toxic product to be colloidal sulphur. This paper is concerned with the inhibitory action of cysteine on the growth of *Mycobacterium tuberculosis* and particularly with the influence of metallic ions on the toxicity of cysteine.

Experimental Procedure

The basal medium for this study was that described by Dubos and Middlebrook (1). In experiments, as noted, casein digest and ferric

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Contribution from the Defence Research Board, Kingston Laboratory, Kingston, Ont.

[2] *Scientific Research Officer.*

[3] *Superintendent.*

ammonium citrate were omitted from the basal medium and cystine or cysteine was added with or without the further addition of salts of iron or the iron group of metals.

In all instances the media, with these omissions or additions, were dispensed in 5 ml. amounts in 6 × 1 in. pyrex test tubes and autoclaved at 15 lb. for 20 min. After autoclaving and cooling, sterile bovine albumin fraction V and glucose were added aseptically as in the standard Dubos and Middlebrook procedure. Throughout the paper this is referred to as *D and M medium* with the omissions or additions stated or as standard *D and M medium* when prepared as described by these authors.

Cultures were inoculated with a calibrated dropping pipette delivering 1/40 ml. from a young culture in standard *D and M medium*. In most instances the culture for inoculum was diluted serially and 1/40 ml. of the diluted culture used.

Cultures

Two strains of *Mycobacterium* have been used. *Mycobacterium tuberculosis* No. 7 is a strain recently isolated from a human case of tuberculosis, which produces progressive tuberculosis in guinea pigs. *Mycobacterium* strain 54 was obtained 20 years ago from the Division of Laboratories, New York State Department of Health, with a history of being one of Koch's original strains of *M. tuberculosis*. At this time Reed and Rice (8) found that it grew rapidly on nutrient agar and was avirulent for guinea pigs. American Type Culture Collection *M. tuberculosis* No. 607 is probably the same culture (4). The culture, with the latter designation, was used by Gottlieb *et al.* (3) in testing antibiotics.

Mycobacterium strain 54 grows luxuriantly in infusion broth or agar. Growth on these media is perceptible in 18 to 24 hr. and luxuriant in 48 hr., though the culture continues to grow for an additional two to three days. The colonies on agar are low convex and smooth to granular but as they mature they become irregular and folded. In *D and M medium* the culture forms a diffuse even growth perceptible in 24 hr. and luxuriant in 48 hr. Quantitative plates may be readily made from these fluid cultures on nutrient agar by the drop-plate method of Reed and Reed (7). The organisms are acid and alcohol fast but long decolorization results in many colorless forms.

Cysteine and Cystine

D and M medium without casein digest or ferric ammonium citrate was supplemented by the addition of 1.0 to 10 mgm. of cystine or 1 to 200 mgm. of cysteine per 100 ml., tubed and autoclaved. Albumen fraction V and glucose were added and the media inoculated with 1/40 ml. of a 1–1000 dilution of a 48-hr. culture of *Mycobacterium* strain 54 in standard *D and M*

medium. As indicated in Table I, cystine up to 10 mgm. per 100 ml., approximately the limit of solubility, and cysteine up to 50 mgm. per 100 ml. had no apparent influence on growth. Higher concentrations of cysteine inhibited growth. The size of the inoculum, however, has a marked influence. When similar cysteine media were inoculated with 1/40 ml. of 1–10, 1–100, and 1–1000 dilution of a 48-hr. culture, growth inhibition was only apparent with the smallest inoculum in the presence of 100 to 200 mgm. of cysteine per 100 ml. as shown in Table II.

TABLE I

GROWTH OF *Mycobacterium* STRAIN 54 IN THE PRESENCE OF CYSTEINE AND CYSTINE ADDED TO *D and M medium* WITHOUT CASEIN DIGEST OR ADDED IRON. INCUBATION 60 HR. AT 37° C.

	Concentration—mgm./100 ml. cystine or cysteine					
	Nil	1	10	50	100	200
Cystine	+	+	+			
Cysteine	+	+	+	+	−	−

\+ = *Growth.*
− = *No growth.*

TABLE II

INFLUENCE OF THE SIZE OF THE INOCULUM ON THE INHIBITION OF GROWTH OF *Mycobacterium* STRAIN 54 IN THE PRESENCE OF CYSTEINE IN *D and M medium* INCUBATION 60 HR. AT 37° C.

Inoculum 1/40 ml., 48-hr. culture	Concentration—mgm./100 ml. of cysteine						
	Nil	20	50	80	100	150	200
1–10	+	+	+	+	+	+	+
1–100	+	+	+	+	+	+	+
1–1000	+	+	+	+	−	−	−

− = *No growth.*
\+ = *Growth.*

In contrast to the above results, when standard *D and M medium* was used containing the usual amounts of ferric ammonium citrate, growth was inhibited by low concentrations of cysteine. Accordingly tests were made on the influence of cysteine and iron in combination.

Ferric ammonium citrate was added to several lots of *D and M medium* to give concentrations from 0 to 50 mgm. per 100 ml. Each lot was again divided and cysteine added to give concentrations from 0 to 100 mgm. per 100 ml. The several lots were then tubed, autoclaved at 15 lb. for 20 min., cooled, and albumin fraction V and glucose added. The media were inoculated with

1/40 ml. amounts of a serial dilution of a 48-hr. culture of *Mycobacterium* strain 54. As shown in Table III growth occurred in all concentrations of cysteine in the absence of iron and in all concentrations of the iron salt in the absence of cysteine. But when 30 mgm. or more of cysteine is added to the medium along with 5 mgm. or more of the iron salt per 100 ml. before autoclaving, growth is inhibited. Such results have been repeatedly obtained.

TABLE III

INFLUENCE OF VARYING CONCENTRATIONS OF CYSTEINE AND FERRIC AMMONIUM CITRATE ON THE GROWTH OF *Mycobacterium* STRAIN 54

Inoculum: 1/40 ml. following dilutions, 48-hr. culture	Concentration ferric ammonium citrate, mgm./100 ml.	Concentrated cysteine—mgm./100 ml.					
		Nil	10	30	50	70	100
1–10	Nil	+	+	+	+	+	+
1–100	Nil	+	+	+	+	+	+
1–1000	Nil	+	+	+	+	+	+
1–10	1	+	+	+	+	+	+
1–100	1	+	+	+	+	+	+
1–1000	1	+	+	+	+	+	+
1–10	5	+	+	−	−	+	+
1–100	5	+	+	−	−	+	+
1–1000	5	+	+	−	−	+	+
1–10	10	+	+	−	−	−	−
1–100	10	+	+	−	−	−	−
1–1000	10	+	+	−	−	−	−
1–10	30	+	+	−	−	−	−
1–100	30	+	+	−	−	−	−
1–1000	30	+	+	−	−	−	−
1–10	50	+	+	+	−	−	−
1–100	50	+	+	+	−	−	−
1–1000	50	+	+	+	−	−	−

− = *No growth.*
\+ = *Growth.*

Similar lots of media containing ferric ammonium citrate and cysteine were inoculated with the virulent *M. tuberculosis* No. 7 from a 10-day culture in *D and M media*. As shown in Table IV growth occurred in all concentrations of cysteine in the absence of iron and in all concentrations of the iron salt in the absence of cysteine. But when 30 mgm. of cysteine was added to the medium along with 1 to 5 mgm. of ferric ammonium citrate per 100 ml. no growth occurred.

TABLE IV

INFLUENCE OF VARYING CONCENTRATIONS OF CYSTEINE AND FERRIC AMMONIUM CITRATE ON THE GROWTH OF *M. tuberculosis* No. 7 AFTER 10 DAYS' INCUBATION

Inoculum: 1/40 ml. following dilutions, 10-day culture	Concentration ferric ammonium citrate, mgm./100 ml.	Concentrated cysteine—mgm./100 ml.					
		Nil	10	30	50	70	100
Undil.	Nil	+	+	+	+	+	+
1–10	Nil	+	+	+	+	+	+
1–100	Nil	+	+	+	+	+	+
Undil.	1	+	+	+	+	+	+
1–10	1	+	+	+	+	+	+
1–100	1	+	+	−	−	−	−
Undil.	5	+	+	−	−	−	−
1–10	5	+	+	−	−	−	−
1–100	5	+	+	−	−	−	−
Undil.	10	+	+	−	−	−	−
1–10	10	+	+	−	−	−	−
1–100	10	+	+	−	−	−	−
Undil.	30	+	+	−	−	−	−
1–10	30	+	+	−	−	−	−
1–100	30	+	+	−	−	−	−
Undil.	50	+	+	−	−	−	−
1–10	50	+	+	−	−	−	−
1–100	50	+	+	−	−	−	−

— = *No growth.*
\+ = *Growth.*

It is apparent that cysteine and iron react to produce a toxic end product.

Other Salts of Iron

The experiment summarized in Table III was repeated with ferric chloride substituted for ferric ammonium citrate to give the same concentration of iron. The results were identical. It is therefore apparent that it is a reaction between the ferric ion and cysteine which provides the growth inhibiting substance.

pH and the Cysteine–Iron Reaction

It was noted that when cysteine and an iron salt were allowed to react in a neutral or alkaline reaction a violet colored compound was formed. It was not formed in acid solutions. This fleeting compound has been described by Michaelis and Yamaguchi (6) as an intermediate cysteine–iron complex. An attempt was made to test its growth inhibiting activity by bringing the reacting substances together in culture media buffered to pH 6.5 to 7.5.

D and M medium was prepared containing 50 mgm. of cysteine per 100 ml. and was divided into three lots to which ferric ammonium citrate was added to give concentrations of 5, 10, and 20 mgm. per 100 ml. Phosphate buffers

were then added in 0.15 *M* concentration to give a pH range from 6.5 to 7.5. The media were autoclaved as usual at 15 lb. for 20 min., albumin fraction V and glucose added, and 5 ml. amounts inoculated with 1/40 ml. of a 1–1000 dilution of a culture of *Mycobacterium* strain 54. As shown in Table V growth was inhibited in media containing 0.05% of cysteine and 5 to 20 mgm. of iron salt at pH 6.5 to 7.0, retarded at pH 7.1, but not inhibited at pH 7.3 and 7.5.

TABLE V

GROWTH OF *Mycobacterium* STRAIN 54 IN *D and M medium* CONTAINING 50 MGM. CYSTEINE PER 100 ML., VARYING AMOUNTS OF FERRIC AMMONIUM CITRATE, AND BUFFERED TO pH 6.5 TO 7.5. INCUBATED 48 HR. AT 37° C.

pH	Concentration ferric ammonium citrate—mgm./100 ml.			
	0	5	10	20
6.5	++	−	−	−
6.7	+++	−	−	−
6.9	+++	−	−	−
7.0	+++	−	−	−
7.1	+++	+	+	+
7.3	+++	+++	+++	+++
7.5	+++	+++	+++	+++

− = *No growth.*
\+ = *Slight amount of growth.*
+++ = *Luxuriant growth.*

Since the violet colored cysteine–iron compound is only formed in neutral or alkaline solutions it is apparent that this is not the growth inhibiting factor. It is also evident that the inhibitor substance results from an iron–cysteine reaction in acid solution.

Cysteine and Metals of the Iron Group

Michaelis and Yamaguchi (6) demonstrated that iron, cobalt, and nickel form related complexes with cysteine in neutral or alkaline solution. *D and M medium* was prepared with 100 to 200 mgm. cysteine per 100 ml. and divided into several lots. Ferric ammonium citrate, nickel chloride, and cobalt chloride were added to provide, in separate lots, 14 to 140 mgm. per 100 ml. The mixtures were buffered to pH 6.8, autoclaved, cooled, glucose and albumin V added, and the mixtures inoculated with 1/40 ml. of a 48-hr. culture of *Mycobacterium* strain 54. Results with iron salt, shown in Table VI, are comparable with the previous finding. The fact that growth was not inhibited in the presence of cysteine and the lower concentration of iron is due to the relatively large inoculum. The higher concentrations of cobalt and nickel

chlorides, in the absence of cysteine, inhibit growth but with the lower concentrations the inhibiting action of the metal salts is partially or completely counteracted by the cysteine.

TABLE VI

GROWTH OF *Mycobacterium* STRAIN 54 IN *D and M medium* CONTAINING VARYING AMOUNTS OF CYSTEINE AND 140 OR 14 MGM. OF SALTS OF IRON, NICKEL, AND COBALT. INCUBATION 48 HR., 37° C.

Concentration metal salt	Concentration cysteine—mgm./100 ml.			
	Nil	100	140	200
Ferric ammonium citrate				
140 mgm./100 ml.	+	−	−	−
14 mgm./100 ml.	+	+	+	+
Nickel chloride				
140 mgm./100 ml.	−	−	−	−
14 mgm./100 ml.	−	−	−	+
Cobalt chloride				
140 mgm./100 ml.	−	−	−	−
14 mgm./100 ml.	−	+	+	+

− = *No growth.*
\+ = *Growth.*

It is therefore evident that, from the point of view of growth inhibition, the reaction between cysteine and iron in acid solution is quite different from the reaction between cysteine and nickel or cobalt salts.

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ANTIBACTERIAL ACTION OF A REACTION PRODUCT OF CYSTEINE AND IRON

II. PREPARATION AND PROPERTIES OF THE SUBSTANCE[1]

By Jack Konowalchuk[2], J. C. Clunie[2], N. A. Hinton[2], and G. B. Reed[3]

Abstract

It has been shown that when low concentrations of cysteine and ferric ammonium citrate or other ferric salts in distilled water, adjusted to faintly acid reaction, are repeatedly autoclaved, an insoluble granular precipitate forms. Chemical analysis of the precipitate indicates that it is sulphur. X-ray photographs of the precipitate and orthorhombic colloidal sulphur, while showing broadly similar patterns, possess significant differences which indicate that the precipitate has a lower symmetry or larger unit cell in its arrangement of sulphur atoms than has orthorhombic sulphur. This precipitate added to Dubos' medium in concentrations of 0.5 to 4 μgm. per ml. inhibits growth of *M. tuberculosis*. In a concentration of 10 μgm. per ml. growth of all of four other Gram positive species of bacteria and three of five Gram negative species was inhibited. Colloidal sulphur had a similar growth inhibiting property but larger concentrations were required than of the precipitate prepared from cysteine and iron.

In the preceding paper, Konowalchuk *et al.* (3), it was shown that when very small concentrations of cysteine hydrochloride and ferric ammonium citrate were added to Dubos and Middlebrook's medium (1) and autoclaved, the medium would not support growth of *Mycobacterium tuberculosis*. Since the cysteine or iron salt when added to the medium singly in the concentrations used had no apparent influence on the growth, it was concluded that growth inhibition resulted from the product or products of a reaction between cysteine and iron.

It has previously been shown that autoclaving cystine or cysteine results in a product toxic to some species of bacteria. Fildes and Richardson (2) found autoclaved cystine solutions to be inhibitory for the growth of *Staphylococcus* and Woiwod (7) considers this to result from the breakdown of cystine to hydrogen sulphide and the formation of colloidal sulphides of copper or other metals. Schuhardt *et al.* (5), on the other hand, found that colloidal sulphur is formed in autoclaved solutions of cystine or cysteine and that the colloidal sulphur is toxic for some species of bacteria.

This paper is concerned with the preparation and properties of the supposed cysteine–iron complex.

Preparation of the Complex

Cysteine and ferric ammonium citrate were dissolved in distilled water, adjusted to a faintly acid solution, autoclaved, cooled, and again autoclaved. The solution on cooling became cloudy and a granular precipitate gradually settled. The precipitate proved to be the growth inhibiting substance.

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Contribution from the Defence Research Board, Kingston Laboratory, Kingston, Ont.
[2] *Scientific Research Officer.*
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After many combinations of cysteine and iron salts and various methods of treatment had been tested, the following procedure was evolved as the best method of producing the complex.

Ten grams of cysteine hydrochloride and 2 gm. of ferric ammonium citrate were added to 20-liter lots of distilled water in 25-liter Pyrex carboys, and sodium hydroxide added to bring the mixture to pH 6.8.

The carboys of solution were then autoclaved at 15 lb. for one hour and allowed to cool overnight and again autoclaved at 15 lb. for one hour. On cooling after the second autoclaving the solution became cloudy and when it had been allowed to stand for several days at 2° to 4° C., a gray white precipitate formed leaving a clear supernatant. The greater part of the clear supernatant was decanted, the remaining suspension centrifuged, and the precipitate washed several times by resuspending in distilled water and centrifuging. Prepared in this manner the material was in the form of small rounded aggregates 1 to 2 μ in diameter and almost completely insoluble in water. Stored as a suspension in water the material very gradually settled out but was readily resuspended on shaking.

The concentration of the complex in suspensions was determined initially by drying to constant weight and weighing. Subsequently it was found more convenient to determine concentrations by measuring opacity in a spectrophotometer. A standard curve was prepared by plotting light transmission of known concentrations against the weight (Fig. 1).

Preliminary trials indicated that the complex, prepared in the above manner, was highly inhibitory to the growth of *Mycobacterium* and other bacteria.

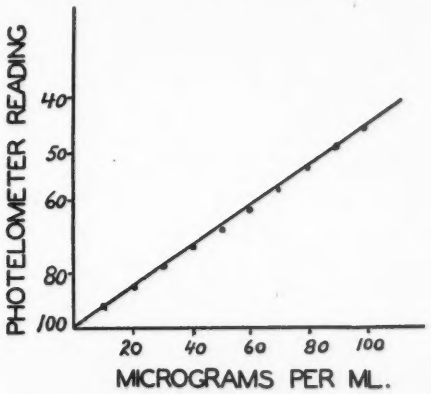

FIG. 1. Standard curve used in estimating the concentration of the complex. Light transmission of a suspension is plotted against weights per milliliter.

Tests of the Complex on Growth of *Mycobacterium*

The complex was added to Dubos and Middlebrook's medium to concentrations of 0.5 to 60 μgm. per ml. The several lots were dispensed in 5-ml. amounts in 6 × 1 in. tubes, bovine albumin fraction V and glucose added as

usual, and the media inoculated with serial dilutions of a 48-hr. culture of *Mycobacterium* strain 54 (3). At the same time drop plate counts were made on the inoculum so that the exact number of viable cells introduced into the complex–containing media was known. The results are shown in Table I, which indicates that when 5-ml. amounts of medium were inoculated with 10 viable cells, 0.5 μgm. of the complex per ml. inhibited growth. With larger inocula higher concentrations of complex were required to inhibit growth, though 4 μgm. per ml. inhibited growth from a relatively large inoculum. Similar results have been repeatedly obtained.

TABLE I

INHIBITION OF GROWTH OF *Mycobacterium* STRAIN 54 AND *M. tuberculosis* BY VARYING CONCENTRATIONS OF THE COMPLEX IN DUBOS' MEDIUM

Concentration of the complex in Dubos' medium, μgm./ml.	Number of organisms in inoculum						
	10^7	10^6	10^5	10^4	10^3	10^2	10
Mycobacterium strain 54							
0 (Control)	+	+	+	+	+	+	+
0.5	+	+	+	+	+	+	−
1	+	+	+	+	+	+	−
4	+	+	+	−	−	−	−
15	+	+	+	−	−	−	−
60	+	+	−	−	−	−	−
M. tuberculosis No. 7							
0 (Control)	+	+	+	+	+	+	+
0.5	+	+	+	+	+	−	−
1	+	+	+	−	−	−	−
4	−	−	−	−	−	−	−
15	−	−	−	−	−	−	−
60	−	−	−	−	−	−	−

\+ = *Growth.*
− = *No growth.*

The experiments were repeated several times with a recently isolated, highly virulent human strain of *M. tuberculosis* No. 7. Dubos and Middlebrook's medium containing 0.5 to 60 μgm. per ml. of the complex, as used in the preceding experiment, was inoculated with decreasing numbers of the tubercle bacilli. In order to inoculate the series of cultures with approximately the same number of viable cells as used in the previous experiment with *Mycobacterium* strain 54, a 14-day culture of *M. tuberculosis* No. 7 in Dubos' medium was adjusted to the same opacity as the diluted strain 54 which

contained 1×10^8 viable cells per ml. It was then assumed that the *M. tuberculosis* culture contained the same number of viable cells. One in 10 serial dilutions were made from this culture and 0.1 ml. of each used as inoculum. Characteristic results, after 10 days' incubation, are indicated in Table I. Control tubes without the complex show abundant growth. Media containing 0.5 μgm. per ml. inhibited growth from the smaller inocula and 4 μgm. inhibited growth from all inocula.

It is also apparent from Table I that *M. tuberculosis* is considerably more sensitive to the action of the complex than the rapidly growing strain 54.

Inhibition of Other Species by the Complex

The growth inhibitory action of the complex was tested on several additional species of bacteria. Dubos and Middlebrook's medium was prepared, containing 10 μgm. of the complex per ml. This was dispensed in 5-ml. lots and inoculated with approximately 10 times the minimum number of cells of each of several species required to initiate growth in Dubos' medium without complex. Table II indicates that of four Gram positive species tested, growth of all is inhibited by 10 μgm. of the complex. Of the five Gram negative species, *Neisseria intracellularis*, *Vibrio cholerae*, *Pasteurella pestis*, and *P. tularense* fail to grow in the presence of the complex, two species of *Salmonella* grow at an apparently normal rate in the presence of 10 μgm. per ml. of the complex.

TABLE II

INHIBITION OF GROWTH OF SEVERAL SPECIES OF BACTERIA BY 10 μGM. OF COMPLEX PER ML. BROTH WHEN INOCULATED WITH APPROXIMATELY 10 TIMES THE MINIMUM NUMBER OF CELLS REQUIRED TO INITIATE GROWTH IN NUTRIENT BROTH

Species	Growth in broth	
	With no complex	With complex, 10 μgm./ml.
Staphyloccus aureus	+	−
Streptoccus pyogenes	+	−
Diplococcus pneumoniae Type II	+	−
Bacillus anthracis	+	−
Neisseria intracellularis	+	−
Vibrio cholerae	+	−
Pasteurella pestis	+	−
Pasteurella tularense	+	−
Salmonella typhosa	+	+
Salmonella typhimurium	+	+

+ = *Growth in 24 hr.*
− = *No growth.*

Influence of Tween 80 on the Inhibitory Action of Complex

In view of the very low solubility of the complex it was anticipated that a wetting agent might influence the activity. As Tween 80 is well known not to be toxic for acid-fast bacteria, it was tried in this connection. Dubos and Middlebrook's medium was divided into two lots to one of which Tween 80 was added to 5% concentration. Each lot was again divided into five parts and complex added to give concentrations of 50, 25, 10, 5, and 0 μgm. per ml. The several lots were dispensed in 5-ml. amounts and inoculated with serial dilutions of a 48-hr. culture of *Mycobacterium* strain 54. It is apparent from the results shown in Table III that the presence of Tween 80 appreciably increases the growth inhibitory action of the complex.

TABLE III

INFLUENCE OF TWEEN 80 ON THE INHIBITION OF GROWTH OF *Mycobacterium* STRAIN 54 BY THE COMPLEX

Dubos' medium	Complex, μgm./ml.	Number of organisms in inoculum				
		10^7	10^6	10^5	10^4	10^3
Without Tween 80	0	+	+	+	+	+
	5	+	+	+	−	−
	10	+	+	−	−	−
	25	+	−	−	−	−
	50	+	−	−	−	−
With 5% Tween 80	0	+	+	+	+	+
	5	+	+	−	−	−
	10	+	−	−	−	−
	25	−	−	−	−	−
	50	−	−	−	−	−

+ = *Growth.*
− = *No growth.*

Analysis of Complex

During autoclaving of the mixture which produced the inhibiting substance, hydrogen sulphide was formed in readily detectable quantities. Schuhardt *et al.* (5) had found small amounts of colloidal sulphur in dilute solutions of cysteine which had been autoclaved, which possibly resulted from the reaction:

$$H_2S + R—S—S—R \longrightarrow S + 2\ RSH\ .$$

The precipitate was therefore tested for this element.

Several samples from various batches of the substance were extracted in a micro-Soxhlet apparatus with Analar grade carbon disulphide with the following results:

Sample	Weight, gm.	% Extracted with CS_2
1	0.0341	102
2	0.1809	96
3	0.0265	97.5
4	0.0733	98
5	0.0450	96.5

In the case of samples 2–5 there remained in the Soxhlet a small quantity of black amorphous residue which was completely inactive *in vitro* against the test organisms. This residue dissolved in concentrated HNO_3/HCl gave the characteristic reactions of ferric iron.

Three samples of the original substance were then analyzed by dissolving them in concentrated HNO_3/HCl plus 1% Br_2 and evaporating repeatedly to dryness to remove HNO_3. The iron was precipitated as $Fe(OH)_3$ and determined as Fe_2O_3. Sulphur was determined gravimetrically as $BaSO_4$. The results fitted the assumption that the complex was a mixture of sulphur and ferrous sulphide. As a further check a sample of the CS_2 extract was evaporated and the residue analyzed as above. The weight of S in the CS_2 agreed within experimental error with the loss in weight of the sample on extraction with CS_2.

X-ray Examination of the Substance

The sulphur precipitated from the autoclaved cysteine/ferric ammonium citrate solutions differed markedly from colloidal sulphur in its antibacterial action. An explanation of this difference was therefore sought in the structure of the two substances.

Dr. T. S. Hutchison of the Royal Military College, Kingston, very kindly examined samples of the two by X-ray diffraction and interpreted the resulting photographs. Debye Scherrer X-ray photographs of the complex and orthorhombic colloidal sulphur are shown together in Fig. 2 for comparison. Low angle lines only appear. The increase in the number of lines in the complex would indicate a lower symmetry or larger unit cell than in the colloidal sulphur. There is, however, a marked similarity between the two patterns. All the orthorhombic lines appear, with altered intensities, in the pattern of complex. It is probable, but not certain, that only minor modifications to the sulphur lattice positions have taken place.

Normally, further X-ray elucidation of the complex lattice would necessitate the preparation of a single crystal. This was not attempted for two reasons,

firstly because the complex is unambiguously recognizable by the powder X-ray method and secondly because there is no guarantee that a prepared single crystal of the complex would have the same structure as the finely divided powder.

Attempts to Prepare Complex by Other Methods

It is probable that the sulphur formed during autoclaving resulted from the oxidation of H_2S either by reaction with cysteine, ferric iron, or atmospheric oxygen, or a combination of all three. Experiments were therefore carried out to try to produce the metastable sulphur by these methods.

Hydrogen sulphide was passed through boiling water at various pH levels containing $FeCl_3$, I_2/KI, O_2, H_2O_2, $KMnO_4$, and $K_2Cr_2O_7$, but only the ordinary colloidal sulphur resulted. The addition of $FeCl_3$ solution and I_2/KI solution to saturated solutions of H_2S at autoclave temperature and pressure produced some colloidal sulphur of the appropriate size but the amounts obtained were too small to permit X-ray examination. Autoclaving ordinary colloidal sulphur produced only a sticky mass on cooling.

Attempts to produce the biologically active sulphur by substituting thioglycollic acid for cysteine in the original process were unsuccessful as were the oxidation methods listed above when sodium thioglycollate was used in place of H_2S.

Colloidal Sulphur

As the analysis of the complex indicated that it is sulphur, a comparison has been made of the activity of complex and colloidal sulphur prepared by conventional methods.

Colloidal sulphur has been shown to have bactericidal activity. Lawson (4) added precipitated sulphur to Cooper's potato medium and reported that 30 mgm. per 100 ml. of medium inhibited the growth of tubercle bacilli. Weld and Gunther (6) found that sulphur dissolved in alcohol when added to culture media inhibited growth of several Gram positive species of bacteria but failed to inhibit Gram negative species. More significant results have been reported by Schuhardt *et al.* (5) who found that 0.06 μgm. per ml. of colloidal sulphur inhibited growth of *Brucella*. More recently Woiwod (7) found that 0.6 to 2 μgm. of similarly prepared colloidal sulphur delayed growth of *Staphylococcus*.

Colloidal sulphur was prepared as follows:

Sulphur	5 gm.
$Ca(OH)_2$	3 gm.
Tween 80	1 drop
Distilled water	500 ml.

The above mixture was boiled for five minutes, filtered, the filtrate neutralized with hydrochloric acid and dialyzed against running water for 48 hr. The end result is a milk white suspension of rounded aggregates.

PLATE I

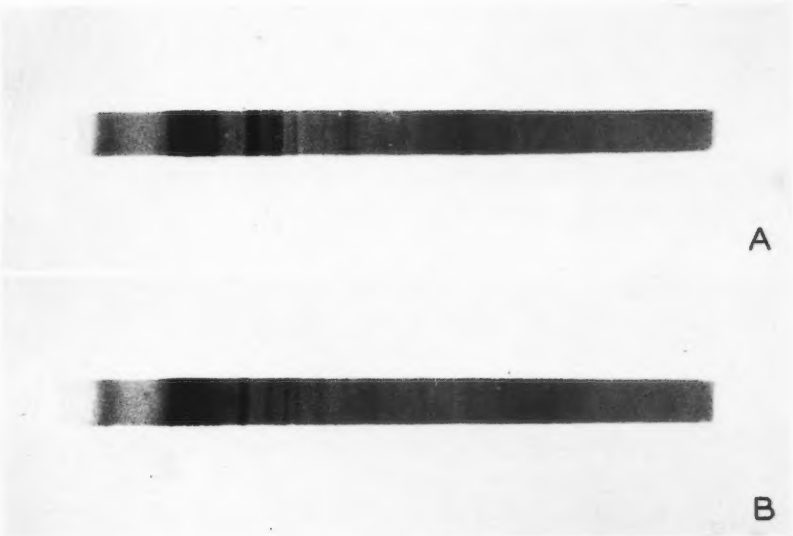

FIG. 2. Debye Scherrer X-ray photographs of "complex" and orthorhombic colloidal sulphur. *A*, the complex, *B*, orthorhombic colloidal sulphur.

The colloidal solution is, however, highly unstable; the sulphur rapidly settles out in crystalline form or as large aggregates which are not broken up on shaking. This is in sharp contrast to the relative stability of the cysteine–iron complex suspension.

The colloidal sulphur, immediately after preparation, was added to Dubos and Middlebrook's medium, autoclaved, glucose and albumin added as usual, and the medium inoculated with serial dilutions of *Mycobacterium* strain 54. For comparison, the complex was added to a second series of the same lot of medium, treated in the same manner, and inoculated with the same culture. The results are shown in Table IV. It is apparent that the complex and colloidal sulphur have a similar inhibitory action on the growth of this organism but the concentration of the complex which is inhibitory is significantly less than that of the colloidal sulphur.

TABLE IV

COMPARISON OF THE INHIBITION OF GROWTH OF *Mycobacterium* STRAIN 54 BY THE COMPLEX AND COLLOIDAL SULPHUR

Complex in Dubos' medium, μgm./ml.	Colloidal sulphur in Dubos' medium, μgm./ml.	Number of organisms in inoculum				
		10^7	10^6	10^5	10^4	10^3
0		+	+	+	+	+
5		+	+	+	−	−
10		+	+	−	−	−
25		+	−	−	−	−
50		+	−	−	−	−
	0	+	+	+	+	+
	5	+	+	+	+	+
	10	+	+	+	+	+
	25	+	+	−	−	−
	50	+	−	−	−	−

+ = *Growth.*
− = *No growth.*

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STUDIES ON THE NUTRITION OF *HELMINTHOSPORIUM SATIVUM* AND CERTAIN RELATED SPECIES[1]

By E. A. Peterson and H. Katznelson

Abstract

Moderate growth of *Helminthosporium sativum* occurred in a simple medium with nitrate, ammonium, or amino nitrogen and was greatly improved by yeast extract. In the presence of a suitable nitrogen source ashed yeast extract also was stimulatory. Growth was markedly increased by a mixture of trace elements but was not influenced by a combination of eight known vitamins. The stimulatory effect of trace elements was noted also with different strains of *H. sativum* and several related species (*H. biforme, H. halodes, H. setariae, H. siccans, H. victoriae*). In the presence of trace elements, several amino acids, especially *l*-proline and *dl*-serine, appeared more favorable for growth of *Helminthosporium* than others tested. Nitrogen source as well as trace elements exerted a distinct effect on type of growth and pigmentation. Zinc appeared to be essential for growth of *H. sativum*; manganese, iron, and boron also may be of importance.

Introduction

The nutrition of *Helminthosporium sativum* has received relatively little attention although this widespread and important fungus pathogen of cereals has been a subject of investigation for many years. Several reports (1, 5, 6) have shown that the fungus is not specific in its nitrogen requirements, and Butler (1) reported that it does not require thiamine or biotin for growth in pure culture. However, it was considered that additional information concerning nutritional requirements would be of value in relation to biological or chemical control and would provide a basis for fundamental studies on sporulation, mutation, pathogenicity, and metabolism. This report deals with the growth response of *H. sativum* and several related species to various nitrogen sources and to trace elements.

Methods

Helminthosporium stock cultures were maintained on potato dextrose agar slants at 26° C. Inoculum consisted of a washed suspension of spores and mycelial fragments prepared from a 10- to 15-day-old culture. The suspension was standardized to provide approximately 1000 spores and fragments per milliliter.

The basal medium, in which the nitrogen source was varied, had the following composition in grams per liter of distilled water: sucrose, 30; KH_2PO_4, 1.0; K_2HPO_4, 1.3; KCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01. Weights of nitrogen compounds used contained the nitrogen equivalent of 2.0 gm. of sodium nitrate, the weights of *dl*-amino acids being doubled.

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Contribution No. 379 from the Bacteriology Division, Science Service, Department of Agriculture, Ottawa, Canada.

Vitamins or trace elements were added in the following concentrations in milligrams per liter: thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, calcium pantothenate, para-aminobenzoic acid, nicotinic acid, each 0.5; inositol, 10.0; biotin, 0.005; $ZnSO_4 \cdot 7H_2O$, 2.0; $MnCl_2 \cdot 4H_2O$, $CuSO_4 \cdot 5H_2O$, $CoCl_2 \cdot 6H_2O$, each 1.6; $NaMoO_4 \cdot 2H_2O$, 1.0; H_3BO_3, 2.4; $CaCl_2$, 1.2.

On the basis of preliminary tests which indicated that best growth of *H. sativum* occurred in the pH range 5 to 7, the reaction of all media was adjusted to pH 6 before sterilization. Heating had no appreciable effect on pH.

All glassware was washed with Alconox* solution, rinsed thoroughly with tap and with distilled water. Cultures were grown at 26° C. in 125 ml. Erlenmeyer flasks; each flask contained 25 ml. of medium and received 1 ml. of inoculum. After 10 days, cultures were filtered through a 200-mesh copper wire screen; the mycelial mats were washed thoroughly with distilled water and excess water was pressed out between two layers of silk. Mats were dried overnight at 85° C. and weighed; averages of duplicate determinations, with ranges indicated, are given in the tables.

No attempt was made to remove trace element contamination from media by special treatment. However, in tests in which trace elements were added, the use of double distilled water was found to decrease variation in yield among replicates.

Results

Nitrogen Utilization

Table I shows that all nitrogen sources tested supported growth of *H. sativum*. Least growth was obtained with *l*-hydroxyproline. The amino acids *dl*-valine, *dl*-norleucine, and *dl*-β-phenylalanine apparently supported best growth, with the exception of yeast extract; however, under the conditions described, mycelial mats produced in the presence of these compounds were rubber-like in consistency, subsurface portions being very gelatinous. A similar type of growth was observed with *dl*-asparagine. The mycelium produced by both *dl*-β-phenylalanine and *dl*-asparagine lacked the characteristic dark pigment; the former induced development of a buff-colored mat, the latter a gray-colored mat. A slimy type of growth was also encountered with ammonium sulphate or ammonium phosphate. Slime production by *H. sativum* may be related to increased acidity. It may be noted also that somewhat improved growth was obtained with *dl*-leucine, *dl*-aspartic acid, and *l*-proline as compared with sodium nitrate. Growth induced by *l*-proline most closely resembled that obtained with yeast extract and on potato dextrose agar, in consequence of which this amino acid was used in many of the subsequent experiments.

* *Manufactured by Alconox Inc., Jersey City 4, N.J.*

TABLE I

GROWTH RESPONSE OF *Helminthosporium sativum* (STRAIN 6) TO VARIOUS SOURCES OF NITROGEN

Nitrogen source	Dry weight of mycelium in mgm.	Final pH
None	0.5 ± 0.0	6.1
$NaNO_3$	22.5 ± 1.9	6.3
$(NH_4)_2SO_4$	17.4 ± 1.2*	2.6
$(NH_4)_2HPO_4$	23.2 ± 2.0*	4.8
Glycine	21.5 ± 1.1	5.9
dl-α-Alanine	20.1 ± 3.5	6.0
dl-Valine	73.1 ± 6.0†	4.8
dl-Leucine	40.0 ± 0.6	4.2
dl-Isoleucine	31.5 ± 0.2	5.3
dl-Norleucine	96.3 ± 3.7†	4.3
dl-β-Phenylalanine	100.1 ± 8.4†	3.8
dl-Tryptophane	18.4 ± 0.1	4.4
l-Cystine	38.0 ± 1.5*	3.9
Cysteine hydrochloride	40.8 ± 1.6*	4.0
dl-Methionine	29.8 ± 0.3	4.2
dl-Aspartic acid	68.0 ± 6.3	6.9
dl-Glutamic acid	23.9 ± 1.6	6.4
l-Arginine hydrochloride	20.6 ± 1.1	5.7
l-Lysine hydrochloride	25.2 ± 0.5	4.0
dl-Asparagine	25.7 ± 2.7	5.5
Citrulline	21.6 ± 0.9	5.5
dl-Ornithine hydrochloride	26.1 ± 0.7	6.1
l-Histidine hydrochloride	32.2 ± 1.4	5.9
l-Proline	40.4 ± 0.4	6.0
l-Hydroxyproline	7.6 ± 0.1	5.9
dl-Serine	20.4 ± 1.0	5.8
dl-Threonine	27.3 ± 2.8	5.5
Casamino acids (vitamin free)	22.6 ± 0.2	5.8
Yeast extract	114.9 ± 4.6	6.2

* *Subsurface slime.*

† *Rubber-like mycelial mats with subsurface slime.*

Influence of Vitamins and Trace Elements

The addition of a mixture of known vitamins to the basal medium containing sodium nitrate or *l*-proline did not replace the growth response obtained with yeast extract. Accordingly, the latter was ashed in order to determine whether its mineral content might be responsible for its growth stimulation. Included as controls were a mixture of vitamins and trace elements, and a mixture of trace elements alone. Table II shows a marked stimulation of growth with ashed yeast extract and with a mixture of trace elements, the effect being more pronounced with *l*-proline than with sodium nitrate. This suggests that the effect of yeast extract was due largely to its mineral rather than its vitamin content, a conclusion which is further supported by the fact that the growth response to a mixture of vitamins and trace elements was not appreciably different from that obtained with trace elements alone.

TABLE II

INFLUENCE OF VITAMINS AND TRACE ELEMENTS ON GROWTH OF TWO STRAINS OF *H. sativum* IN LIQUID CULTURE

Nitrogen source	Supplements	Dry weight of mycelium in mgm.	
		Strain 6	Strain 638
$NaNO_3$	None	21.5 ± 0.3	11.6 ± 0.1
$NaNO_3$	Vitamins	25.7 ± 2.2	18.0 ± 0.0
$NaNO_3$	Ashed yeast extract	47.2 ± 6.2	89.8 ± 4.5
$NaNO_3$	Trace elements	73.0 ± 0.7	93.0 ± 3.3
$NaNO_3$	Trace elements + vitamins	89.2 ± 2.3	85.4 ± 4.9
l-Proline	None	17.2 ± 0.8	17.9 ± 1.6
l-Proline	Vitamins	23.7 ± 0.5	26.6 ± 1.3
l-Proline	Ashed yeast extract	113.1 ± 5.9	80.7 ± 8.2
l-Proline	Trace elements	185.4 ± 2.9	118.2 ± 8.2
l-Proline	Trace elements + vitamins	158.0 ± 2.6	109.3 ± 3.7
Yeast extract	None	111.5 ± 4.4	103.1 ± 1.8

Influence of Nitrogen Source and Trace Elements on Growth of Different Strains of Helminthosporium sativum and Several Related Species

Since the addition of trace elements to the basal medium containing either sodium nitrate or *l*-proline stimulated growth of *H. sativum*, the trace element response of different strains of the fungus and several other species was also studied. *dl*-Asparagine, *dl*-glutamic acid, *l*-proline, and *dl*-serine were selected for these tests from 23 amino acids on the basis of highest mycelial yields in the presence of added trace elements. *dl*-α-Alanine and *dl*-β-phenylalanine were also included in the experiment on species response. Results for strains and species are summarized in Tables III and IV respectively. Although both

TABLE III

GROWTH RESPONSE OF FIVE STRAINS OF *H. sativum* TO DIFFERENT NITROGEN SOURCES AND THE INFLUENCE OF TRACE ELEMENTS

Nitrogen source	Supplement	Dry weight of mycelium in mgm.				
		Strain 2	Strain 5	Strain 6	Strain 638	Strain W
$NaNO_3$	None	28.4 ± 0.9	27.4 ± 2.2	20.8 ± 1.3	16.4 ± 0.3	31.8 ± 3.4
$NaNO_3$	Tr. elements	168.8 ± 12.3	65.1 ± 2.6	56.5 ± 1.6	108.4 ± 3.2	84.6 ± 1.8
dl-Asparagine	None	43.5 ± 0.7	42.4 ± 2.6	25.7 ± 2.7	26.1 ± 1.3	42.3 ± 2.0
dl-Asparagine	Tr. elements	241.0 ± 2.9	277.2 ± 5.1	219.8 ± 2.1	169.6 ± 8.9	265.6 ± 7.8
dl-Glutamic acid	None	17.9 ± 3.9	25.5 ± 1.9	23.9 ± 1.6	11.8 ± 0.1	17.7 ± 1.4
dl-Glutamic acid	Tr. elements	206.9 ± 6.2	165.0 ± 4.6	122.2 ± 5.2	79.0 ± 0.4	143.7 ± 2.7
l-Proline	None	33.1 ± 2.1	36.5 ± 1.3	40.4 ± 0.4	25.4 ± 0.6	28.8 ± 1.6
l-Proline	Tr. elements	238.9 ± 5.4	214.1 ± 3.0	214.2 ± 6.4	153.0 ± 0.4	207.5 ± 1.2
dl-Serine	None	45.8 ± 3.0	41.3 ± 5.3	20.4 ± 1.0	32.5 ± 3.5	45.3 ± 11.9
dl-Serine	Tr. elements	296.0 ± 10.9	302.3 ± 1.7	101.7 ± 4.1	120.3 ± 0.3	102.6 ± 8.9
Yeast extract	None	146.7 ± 1.7	136.8 ± 1.9	114.9 ± 4.6	108.0 ± 2.6	131.9 ± 0.9
Yeast extract	Tr. elements	168.5 ± 4.8	174.9 ± 2.7	111.6 ± 4.6	118.9 ± 0.2	153.5 ± 3.2

TABLE IV

GROWTH RESPONSE OF FIVE SPECIES OF *Helminthosporium* TO DIFFERENT NITROGEN SOURCES AND THE INFLUENCE OF TRACE ELEMENTS

Nitrogen source	Supplement	Dry weight of mycelium in mgm.				
		H. biforme	*H. halodes*	*H. setariae*	*H. siccans*	*H. victoriae*
$NaNO_3$	None	16.9 ± 1.7	42.7 ± 3.4	24.3 ± 2.1	13.6 ± 2.5	41.0 ± 1.7
$NaNO_3$	Tr. elements	30.1 ± 0.5	305.3 ± 1.2	217.9 ± 2.8	77.4 ± 11.8	214.9 ± 16.3
dl-α-Alanine	None	34.8 ± 8.4	62.4 ± 8.3	35.7 ± 3.6	19.2 ± 0.8	66.5 ± 4.1
dl-α-Alanine	Tr. elements	174.1 ± 10.9	385.2 ± 9.1	353.5 ± 4.7	354.2 ± 1.8	396.8 ± 10.7
dl-Asparagine	None	57.4 ± 1.7	65.4 ± 2.4	51.6 ± 7.1	25.0 ± 3.5	91.1 ± 5.4
dl-Asparagine	Tr. elements	309.3 ± 10.9	353.4 ± 2.1	332.7 ± 4.2	339.0 ± 12.0	262.9 ± 3.9
dl-Glutamic acid	None	17.9 ± 0.1	47.0 ± 6.5	23.3 ± 1.6	19.8 ± 2.4	64.0 ± 9.3
dl-Glutamic acid	Tr. elements	54.6 ± 3.5	380.4 ± 4.8	100.7 ± 4.4	172.4 ± 0.4	222.6 ± 3.1
dl-β-Phenylalanine	None	36.4 ± 0.4	135.8 ± 4.1	31.5 ± 1.1	5.4 ± 0.7	74.4 ± 9.7
dl-β-Phenylalanine	Tr. elements	108.3 ± 7.2	375.4 ± 5.2	81.6 ± 2.7	3.5 ± 0.3	86.2 ± 1.4
l-Proline	None	30.8 ± 4.8	86.0 ± 0.9	65.5 ± 7.6	44.1 ± 1.2	119.8 ± 9.3
l-Proline	Tr. elements	242.1 ± 5.1	366.4 ± 1.4	260.5 ± 1.0	209.0 ± 6.5	302.0 ± 1.9
dl-Serine	None	47.6 ± 2.8	87.4 ± 1.2	41.8 ± 0.3	35.7 ± 3.6	78.2 ± 13.2
dl-Serine	Tr. elements	304.8 ± 11.6	361.8 ± 2.4	334.4 ± 9.8	349.4 ± 0.7	335.4 ± 4.2
Yeast extract	None	227.8 ± 0.4	218.9 ± 0.9	168.1 ± 0.1	158.8 ± 7.6	168.7 ± 2.7
Yeast extract	Tr. elements	233.8 ± 2.7	271.9 ± 1.4	185.6 ± 4.1	164.4 ± 3.5	142.0 ± 0.4

strain and species variation in response to different nitrogen sources may be noted, the data indicate clearly the importance of trace elements in the nutrition of *Helminthosporium*. *dl*-Asparagine and *l*-proline appeared most generally favorable for growth of all strains of *H. sativum*, whereas *dl*-serine was favorable only for strains 2 and 5. However, it was noted that cultures of all strains grown in the medium containing *dl*-asparagine differed from those grown in the presence of the other nitrogen sources in that they lacked the dark pigment commonly associated with growth of this fungus. In all strains, *dl*-asparagine induced development of a gray, rubber-like mat the subsurface of which was gelatinous with clumps of white aerial mycelium on the surface.

Table IV shows that *dl*-β-alanine, *dl*-asparagine, *l*-proline, and *dl*-serine, with added trace elements, were superior to yeast extract at equivalent nitrogen levels for most of the species tested. One species, namely *H. siccans*, was unable to utilize *dl*-β-phenylalanine although moderate to excellent growth of the remaining species was obtained with this compound. Several species, especially *H. halodes*, produced abundant growth even with sodium nitrate in the presence of trace elements. Microscopic examination of scrapings from mycelial mats indicated that sporulation of *H. biforme* and *H. victoriae* was markedly increased by the addition of trace elements to media containing *dl*-asparagine, *l*-proline, *dl*-serine, or *dl*-α-alanine as sources of nitrogen.

The Effect of Omission of Individual Trace Elements

Preliminary studies on the effect of omitting individual elements from a mixture on growth of *H. sativum* were conducted with sodium nitrate and *l*-proline as nitrogen sources. Results summarized in Table V show a marked reduction in mycelial yield in the absence of zinc. Lack of manganese, iron, or boron also depressed growth but to a less marked extent. Growth obtained when all eight elements were omitted indicates trace element contamination of the medium.

TABLE V

GROWTH RESPONSE OF *H. sativum* TO THE OMISSION OF INDIVIDUAL TRACE ELEMENTS FROM A MIXTURE

Element omitted	Dry weight of mycelium in mgm.	
	$NaNO_3$	*l*-Proline
None	40.5 ± 1.6	176.9 ± 9.6
Fe	29.2 ± 3.4	143.5 ± 0.8
Zn	8.1 ± 0.6	11.2 ± 0.2
Mn	17.9 ± 1.7	117.8 ± 10.2
Cu	51.6 ± 11.1	121.0 ± 1.6
Co	39.7 ± 5.7	160.7 ± 9.3
Mo	34.0 ± 0.1	157.0 ± 0.1
B	30.3 ± 0.4	146.9 ± 2.8
Ca	35.7 ± 2.9	163.5 ± 9.5
All	18.4 ± 1.9	30.8 ± 3.5

Discussion

All the nitrogen compounds tested supported varying degrees of growth of *H. sativum* in liquid media but none approached the growth response induced by yeast extract when supplied at an equivalent nitrogen level. The fact that ashed yeast extract significantly stimulated fungus growth indicates the importance of inorganic elements in the nutrition of *H. sativum*. While yeast extract also supplies various growth factors, these appear unimportant to the fungus since the addition of known vitamins gave no appreciable growth stimulation over that obtained in controls. Similar results have been reported by Converse (2) for *H. gramineum* and by Elliott (3) who found *H. gramineum* and *H. victoriae* to be self-sufficient for certain vitamins. In the presence of an adequate supply of trace elements, certain amino acids, especially *l*-proline and *dl*-serine, were found to be nutritionally superior to other nitrogen sources tested. Converse (2) has suggested a possible relationship between growth response of *H. gramineum* to specific amino acids such as *l*-tyrosine and *l*-proline in culture and its invasion of the host.

Preliminary results indicate that zinc is probably essential for *H. sativum*; manganese, iron, and boron may be of importance also. However, optimum concentrations were not established and media received no special treatment to remove trace element contamination.

Foster (4) has emphasized the importance of trace elements in the nutrition of fungi in relation to morphological and cytological characteristics, chemical composition, sporulation, and pigmentation. During the present studies it was noted that the nitrogen source as well as the trace elements exerted a distinct effect on the type of growth and pigmentation. Trace elements also appeared to favor sporulation of *H. biforme* and *H. victoriae*. These observations point to the desirability of further studies on the nutrition of *H. sativum*.

Acknowledgment

The authors gratefully acknowledge the technical assistance of Miss Margaret I. Chisholm.

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THE METABOLISM OF SEVEN-CARBON SUGARS, ACIDS, AND ALCOHOLS BY BACTERIA AND YEASTS[1]

By W. B. Moore[2], A. C. Blackwood, and A. C. Neish

Abstract

The utilization of heptoses and related compounds by bacteria and yeasts was investigated. Most cultures metabolized sedoheptulose, which was the only sugar dissimilated under both aerobic and anaerobic conditions. No other heptuloses were utilized by these organisms. Some cultures were able to oxidize various heptoses to the corresponding heptonic acids and certain *Acetobacter* strains produced other compounds. Heptitols were attacked by only the *Acetobacter* species. Heptonic acids were utilized slightly by a few organisms.

Introduction

The importance of sedoheptulose as an intermediate in photosynthesis and in pentose metabolism by plant and animal tissues and by bacteria is now well established. However, little is known of the metabolism of other seven-carbon sugars and related compounds. Some of these compounds have been previously surveyed as substrates for bacteria by measurement of acid and gas production (10, 20), or growth (8). Sternfeld and Saunders (20) report no utilization of D-gluco-D-guloheptose (D-α-glucoheptose) by 17 bacteria and two yeasts and Koser and Saunders (10) reported negative utilization for this heptose and D-glucoheptulose. However, Johnson and Schwarz (8) found that *Bacterium aliphaticum* grew on three of four heptoses and one of two heptuloses tested. Hudson and co-workers (5, 12, 21) studied the activity of *Acetobacter* species on various heptitols, and isolated heptuloses in good yields after fermentation.

Roe and Hudson (17, 18) found that D-mannoheptulose administered either orally or parenterally to rabbits is metabolized to a copper-reducing compound which was fermentable by yeast. Rats cannot metabolize this heptulose. Neither rats nor rabbits can metabolize D-manno-D-galaheptose.

The aim of this investigation was to find which bacteria or yeasts metabolize seven-carbon sugars and related compounds; which of these compounds are most readily utilized; and to identify the products formed.

Materials and Methods

Cultures

All of the organisms were taken from our culture collection and most of these were named-cultures obtained previously from elsewhere on request. A few organisms that rapidly utilize some of these heptoses under aerobic conditions were isolated from soil by enrichment techniques but no organisms

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[2] *National Research Council Postdoctorate Fellow, 1952–54.*

were obtained which would metabolize anaerobically any seven-carbon sugar except sedoheptulose. One of these isolates was identified as *Aerobacter aerogenes*, three as *Pseudomonas aeruginosa*, and two were unidentified.

Preparation of the Seven-Carbon Compounds

The compounds used in this investigation are listed in Table I, together with references to the methods of preparation. The preparation of D-altro-heptulose (sedoheptulose) was modified for greater convenience. The plant material (*Sedum spectabile* Bor.) was frozen, mixed with 40% ethanol in a Waring blendor, heated to boiling, filtered with celite, evaporated under reduced pressure, treated with baker's yeast for a few hours, treated with lead acetate, filtered, deionized by IR-120 and IR-4B Amberlite resins, treated with charcoal, and finally concentrated (below 40° C.) to a colorless sirup. Paper chromatography showed sedoheptulose to be the major component although several minor impurities were demonstrated (see Fig. 1).

All sodium amalgam reductions were carried out in oxalate buffered media (pH about 3.5). Other modifications of the original methods were made when the use of ion-exchange resins was advantageous for removal of salts or replacement of metal cations by hydrogen ions.

TABLE I

PREPARATION OF SEVEN-CARBON COMPOUNDS

Compound	Method of preparation	Reference
Heptoses		
D-Gluco-D-gulo	Reduction of lactone by $NaBH_4$	(23)
L-Gala-D-gluco	Reduction of lactone by sodium amalgam	(1)
L-Gala-D-manno	Reduction of lactone by sodium amalgam	(1)
D-α-Fructo	Reduction of lactone by sodium amalgam	(24)
Heptuloses		
D-Gluco	From D-gluco-D-guloheptose and alkali	(2)
D-Manno	Isolated from avocado fruit	(13)
D-Altro*	Isolated from *Sedum spectabile*	(11)
Heptitols		
Gluco-gulo	Reduction of lactone by $NaBH_4$	(22)
D-Manno-D-gala†	Isolated from avocado fruit	(13)
D-α-Fructo	Catalytic hydrogenation of corresponding aldose	(24)
Heptonic acids		
D-Gluco-D-gulo‡ D-Gluco-D-ido	From D-glucose by cyanhydrin synthesis	(6)
D-Gala-L-manno	From D-galactose by cyanhydrin synthesis	(3, 4)
D-Manno-D-gala D-Manno-D-talo	From D-mannose by cyanhydrin synthesis	(7)
D-α-Fructo§	From D-fructose by cyanhydrin synthesis	(24)

* *Sedoheptulose.*
† *Perseitol.*
‡ *The lactone and calcium salt are commercially available (Pfanstiehl).*
§ *This is believed to be 2-C-hydroxymethyl-D-gluconic acid (25).*

Fermentation Conditions

As the supply of compounds was limited, only small amounts were fermented. The basal medium contained 0.5% yeast extract (Difco), 0.5% substrate, 0.02% glucose (in most trials), and bromcresol purple. The medium was distributed in 1-ml. portions in small test tubes and sterilized by autoclaving at 121° C. for 10 min. Inocula were grown in a medium containing 0.5% yeast extract and 0.5% glucose for 24 hr. at 30° C. and one drop (*ca.* 0.02 ml.) was transferred to each tube. The tubes were then placed on a rotary shaker and incubated for 14 days at 30° C. Tubes inoculated with anaerobes were placed in a desiccator which was evacuated and filled with oxygen-free nitrogen three times, before it was placed on the shaker. The fermentation of glucose was included as a control for each culture in the survey.

Analytical Methods

Following incubation the acidity of each tube was checked, then the contents were evaporated to dryness in a current of warm air at 45°–60° C. (paper chromatography of controls indicated that this caused little change of the substrates). The residue was then made to volume.

Reducing sugars were determined by the method of Somogyi (19) and color developed with the Nelson reagent (15). The reagent was standardized against each sugar. Sugar acids and alcohols were determined by the measurement of the formaldehyde formed on periodate oxidation (14).

When utilization of the substrate was found the fermentation liquor was analyzed by descending paper chromatography. Whatman No. 1 paper was irrigated for 18–24 hr. at 30° C. with the upper phase of an *n*-butanol–ethanol–water mixture (40 : 10 : 50). Compounds on the chromatograms were revealed with silver nitrate (22) and reducing sugars indicated with aniline phthalate (16) or aniline malonate (9).

Results

Heptoses

The utilization of the four heptoses is given in Table II for 73 cultures. *Bacillus* species did not metabolize these heptoses to any extent although many organisms utilized L-gala-D-mannoheptose. The negative result was also found in an additional trial with 39 more bacilli grown in the presence of D-gluco-D-guloheptose, but *B. cereus* and *B. polymyxa* cultures gave positive V.P. tests for acetoin although little substrate was used and no acid or gas appeared. Note that *B. macerans* cultures give an acid reaction with some of these heptoses (Table II).

The *Clostridium* species, however, utilized three of the heptoses (not L-gala-D-manno), but not completely in any instance.

Aerobacter aerogenes, *Serratia marcescens*, and some *Pseudomonas* species gave the best utilization of the heptoses. Paper chromatography showed that these organisms converted the heptose to one or two main compounds. This was later found to be a result of lactonization of the various acids causing

TABLE II

SURVEY OF BACTERIA AND YEASTS FOR THE UTILIZATION OF SEVEN-CARBON SUGARS*

(The results are given as the number of cultures metabolizing 20% or more of the substrate. The average per cent of substrate utilized is given for the positive cultures. An "A" indicates that one or more cultures gave an acid reaction.)

Culture	No. strains tested	Heptose								Heptulose			
		D-Gluco-D-gulo		D-α-Fructo		L-Gala-D-gluco		L-Gala-D-manno		D-Altro		D-Gluco	
			%		%		%		%		%		%
Bacillus subtilis	4	0		0		0		4	30	4	30	0	
Bacillus subtilis var. *aterrimus*	1	0		0		0		1	24	1	30	0	
Bacillus subtilis var. *niger*	1	0		0		0		1	25	1	100	0	
Bacillus subtilis var. *globigii*	1	0		0		0		0		1	96	0	
Bacillus licheniformis	5	0		0		2	27	5	22	5A	76	0	
Bacillus pumilus	2	0		0		1	20	2	27	2	100	0	
Bacillus megaterium	2	0		0		0		1	27	2	100	0	
Bacillus cereus	2	0		0		0		0		0		0	
Bacillus sphaericus	2	0		0		0		0		0		0	
Bacillus alvei	1	0		0		0		1	21	1	24	0	
Bacillus para-alvei	1	0		0		0		0		0		0	
Bacillus laterosporus	1	0		0		0		0		0		0	
Bacillus brevis	1	0		0		0		0		0		0	
Bacillus circulans	1	0		0		0A		0		0		0	
Bacillus coagulans	1	0		0		0		0		1	30	0	
Bacillus polymyxa	2	0		0		0		1	20	2	24	0	
Bacillus macerans	2	0		0A		0A		0A		0A		0	
Clostridium acetobutylicum	1	1	29	1	34	1	31	0		0		1	29
Clostridium pasteurianum	1	1	27	1	30	1	30	0		1	100	1	30
Clostridium sp.	1	0		1	34	1	34	0		0		1	30
Aerobacter aerogenes	3	2A	88	3A	96	0A		3	22	3	97	0	
Escherichia coli	2	0		0		0		0		1	98	0	
Erwinia carotovora	2	0		0		0		0		1	97	0	
Proteus vulgaris	2	0		0		0		0		2	83	0	
Serratia marcescens	1	1A	78	1	100	0A		0		1	99	0	
Serratia anolium	1	1A	47	1A	100	0A		0		1	98	0	
Serratia plymouthensis	1	0		0		0		0		1	100	0	
Serratia kielensis	1	0		0		0		0		1	71	0	
Salmonella gallinarum	1	0		0		0		1	31	0		0	
Pseudomonas aeruginosa	4	4A	90	4A	89	0A		4A	35	0		0	
Pseudomonas coronafaciens	1	1	26	1A	100	0		0		0		0	
Pseudomonas fluorescens	1	1	95	1A	100	0A		1	33	0		0	
Pseudomonas hydrophila	2	0		0		0		0		1	97	0	
Xanthomonas translucens	1	0		1	85	0		0		1	69	0	
Acetobacter suboxydans	2	0A		0A		1A	52	0A		1A	27	0	
Acetobacter xylinum	2	1A	42	1A	85	0		0		2	26	0	
Acetobacter aceti	2	0		1A	100	0A		1	30	0		0	
Acetobacter rancens	2	1A	55	1A	87	0		0		0		0	
Acetobacter acetosum	1	0		1A	48	0		1	22	0		0	
Agrobacterium tumefaciens	1	0		0		0		1	21	0		0	
Corynebacterium flaccumfaciens	1	0		0		0		0		1	97	0	
Mycobacterium smegmatis	1	0		0		0		0		1	96	0	
Brucella bronchiseptica	1	0		1	88	0		1	20	1	96	0	
Micrococcus pyogenes	1	0		1	88	0		1	23	1	96	0	
Saccharomyces cerevisiae	1	0		0		0		0		1	100	0	
Zygosaccharomyces richteri	1	0		0		0		1	20	0		0	
Torula pulcherima	1	0		0		0		1	21	0		0	

* *See text for results with heptitols and heptonic acids.*

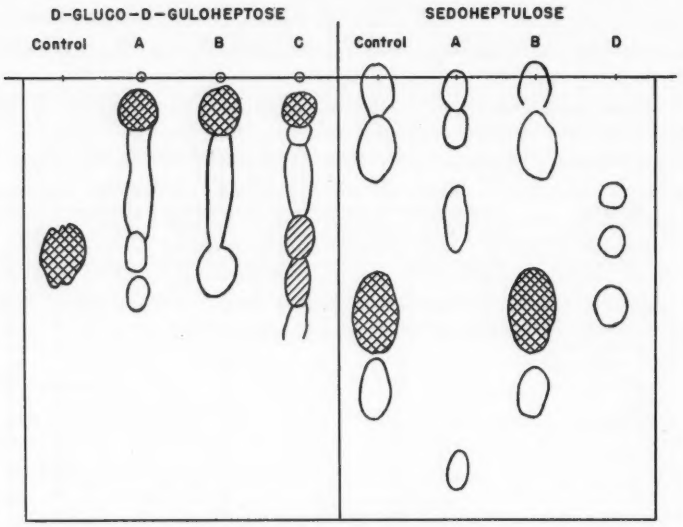

FIG. 1. Schematic tracing of representative chromatograms of the fermentation of seven-carbon sugars by various organisms:
(*a*) *Aerobacter aerogenes*, (*b*) *Pseudomonas fluorescens*,
(*c*) *Acetobacter xylinum*, (*d*) *Saccharomyces cerevisiae*.
Spray—alkaline silver nitrate.

two main spots and often a connecting streak. Representative chromatograms are given in Fig. 1. When the heptose is utilized, conversion to the corresponding heptonic acid takes place by all organisms tested except some of the *Acetobacter* species (Fig. 1). The products formed by this latter group are as yet unidentified.

In one trial, 2.5 gm. of D-gluco-D-guloheptose was oxidized completely by *Aerobacter aerogenes*, R_4, after 10 days' incubation at 30° C. Paper chromatographic analysis indicated that only the heptonic acid was formed and analysis with periodic acid showed that the conversion was quantitative. The product was isolated by removal of cells and soluble protein with zinc hydroxide (14); removal of cations on a column of Amberlite IR-120 resin; absorption of the acid on a column of Amberlite IR-4B resin and elution with normal sulphuric acid. Paper chromatographic analyses of eluate fractions showed that both free acid and lactone were present. Then, the combined fractions were decolorized with charcoal, concentrated at low temperature, and the crystalline lactone obtained by addition of ethanol and storage at 0° C. overnight. The melting point and mixed melting point of this material with authentic D-gluco-D-guloheptonic lactone (m.p. 149–150° C.) were identical.

Heptuloses

D-Altroheptulose (sedoheptulose) is utilized by many of these cultures, the notable exception being *Pseudomonas aeruginosa* and related species (Table II).

Additional trials with *Aerobacter aerogenes* have shown that under anaerobic conditions utilization is rapid and complete. Paper chromatographic analyses showed that a variety of compounds were formed (Fig. 1). *Saccharomyces cerevisiae* was found in repeated trials to ferment this sugar (Table II). LaForge and Hudson (11) first discovered sedoheptulose when they found reducing sugar remaining after a yeast fermentation of plant juice from *Sedum spectabile*, and they isolated and identified the sugar. Probably an adaption period in the absence of other fermentable sugars is necessary for the yeast to utilize this heptulose.

No other heptulose was attacked although the *Clostridium* species appear to have some activity on D-glucoheptulose (Table II). D-Mannoheptulose was tested against 39 other strains of bacilli with negative results. In this latter test none of the *B. para-alvei* strains would grow in the presence of this heptulose although growth occurred with all other cultures and with these strains on other substrates.

TABLE III

UTILIZATION OF HEPTONIC ACIDS BY BACTERIA

(The cultures using the acids are listed with the per cent substrate metabolized in brackets)

Heptonic acid	Culture
D-Gluco-D-gulo	None
D-Gluco-D-ido	*B. coagulans*, B35 (20%)
D-Gala-L-manno	*B. pumilus*, B48 (34%); *B. globigii*, B467 (31%)
D-Manno-D-gala	*B. globigii*, B467 (30%); *B. pumilus*, B48 (27%); *B. licheniformis*, B478 (29%), B2 (46%)
D-Manno-D-talo	*B. licheniformis*, B453 (40%); *B. globigii*, B467 (59%)

Heptitols

Of the 73 cultures listed in Table II only *Acetobacter suboxydans* attacked gluco-gulo heptitol and D-α-fructoheptitol. Chromatography and analyses for reducing sugar indicated that a sugar was the main product, undoubtedly L-glucoheptulose from gluco-gulo heptitol as has been reported previously (12). D-Manno-D-galaheptitol (perseitol) was checked against 70 bacilli with negative results.

Heptonic Acids

D-Gluco-D-gulo- and D-α-fructo-heptonic acids were not attacked by any of the cultures listed in Table II. Five heptonic acids were tested against 70 bacilli but the products formed were not identified (Table III). Strains of *B. sphaericus*, although unable to utilize any sugar, appeared to dissimilate slowly some of the heptonic acids producing reducing compounds. These also were not identified.

Discussion

No pattern of heptose metabolism was established between groups of bacteria or between species except in a few instances. Some of the *Pseudomonas* species differ from other organisms in that the heptoses are oxidized but sedoheptulose is not fermented. The exception is *Pseudomonas hydrophila*, which in many biochemical activities is not typical of other pseudomonads. While certain cultures can metabolize these heptoses, closely related organisms cannot, and strain variation appears to be more important than species or other taxonomic differentiation although certain genera are more active on these heptoses. It is difficult to relate the results obtained to the structural configuration of the sugars and no pattern of metabolism is apparent.

The production of acid in the medium is not a good indication of substrate utilization. For example, *B. macerans* gave an acid reaction when supplied with certain heptoses although no significant amount of substrate was utilized while many cultures gave no acid reaction although the substrate was metabolized. Even though the heptose was converted to heptonic acid there was frequently no visible change in the pH indicator. For this reason, although Sternfeld and Saunders (20) found no change in the pH indicator, certain bacteria undoubtedly oxidized D-gluco-D-guloheptose to the heptonic acid. A chemical estimation of substrate utilized is necessary in this type of survey if authentic results are to be obtained with a wide range of organisms.

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BIOLOGICAL OXIDATION OF NAPHTHALENE[1]

By R. J. Strawinski[2] and R. W. Stone

Abstract

Conditions have been established for the oxidation of naphthalene by a pseudomonad based on the yields of ether extractable substances. Vigorous aeration yielded approximately three times the product obtained from unaerated controls. Oxidations with media adjusted initially to pH 8 gave higher yields than those adjusted to lower pH levels. Calcium and copper appeared to be necessary for maximum yields. Under the conditions described, the non-naphthalenic ether extractable substances amounted to as much as 29% of the original 1% naphthalene. Purification and analysis of the crude extract showed that it consisted largely of salicylic acid. Some properties of the remaining material are described.

In view of the increasing amount of work on the oxidative pathways of aromatic compounds (8, 11) it is of interest to examine the mechanism of naphthalene dissimilation. There are several early papers showing that certain bacteria can utilize naphthalene as a sole carbon source, such as represented by Tausson (10), Tattersfield (9), and Gray and Thornton (3). None of these workers reported the identification of any products from the biological oxidation.

Jacobs (4) obtained a positive fluorescein test for phthalic acid in a bacterial culture grown on naphthalene. In 1943 Strawinski and Stone (7) published an abstract stating that salicylic acid was one of the main products resulting from the oxidation of naphthalene by a pseudomonad. This work was interrupted by World War II and is now reported here. Recently, Walker and Wiltshire (11) showed that salicylic acid was formed from the dissimilation of naphthalene by a strain of *Pseudomonas*. They isolated and identified D-*trans*-1,2-dihydro-1,2-dihydroxynaphthalene. The formation of naphthalene diols previously had been demonstrated in animal metabolism of naphthalene by Young (12) and Booth and Boyland (2).

A study of oxidation of naphthalene by members of the *Pseudomonas* group was initiated to determine conditions under which naphthalene was most readily attacked and which would provide significant amounts of products in addition to bacterial cells and carbon dioxide.

Experimental Methods

The basal medium employed was composed of NH_4NO_3, 0.25 gm.; KH_2PO_4, 0.05 gm.; Na_2HPO_4, 0.10 gm.; $MgSO_4$, 0.05 gm.; $MnCl_2$, 0.02 gm.; $CaCl_2$, 0.005 gm.; $FeCl_2$, 0.005 gm.; KI and $CuSO_4 \cdot 5H_2O$, trace; 100 ml. tap water. The pH after autoclaving at 120° C. for 15 min. was about 6.5. Any adjustment of pH was made after sterilization. Sterile, finely pulverized

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naphthalene was added in a concentration of 1% by weight after autoclaving. All incubation was conducted at 28° C. A 5% inoculum of a seven-day-old culture was used unless otherwise noted. The volume of medium in 2-liter Erlenmeyer flasks was either 750 or 800 ml.; 250-ml. amounts were used in liter flasks.

Cultures capable of oxidizing naphthalene were obtained by an enrichment technique using the basal medium plus naphthalene. Fifty-milliliter portions in 250-ml. flasks were inoculated with 5-gm. samples of garden soil and allowed to incubate with occasional (three or four times per day) shaking by hand. Subcultures were made at 14-day intervals. After 11 transfers nutrient agar plates streaked from the naphthalene liquor showed predominantly one type of organism. Several strains were picked and one was selected for study.

On completion of incubation the media were chilled and filtered to remove the insoluble naphthalene and mineral salts. The residues were washed with water and washings added to the respective filtrates. The culture liquors were then made acid to Congo red with hydrochloric acid and continuously extracted with ether for 72 hr. Ether was removed from the extracts under reduced pressure and the product was dried and weighed.

When sufficient product for chemical analysis was desired, the oxidation of naphthalene was conducted in 10-liter batches. A 12-liter flask was fitted with a fused alumina gas-diffuser stone through which sterile water-saturated air was passed in sufficient amount to obtain vigorous agitation and aeration. The larger batches of liquor were neutralized before filtration and concentrated to one half volume under vacuum before extraction.

Results

Description of Culture

The culture used in this study produced a water-soluble green pigment on nutrient agar and in broth. The organism was a Gram-negative rod, motile with one polar flagellum. It produced acid but no gas from glucose broth, pyocyanin from asparagin broth, reduced nitrate to nitrite, showed rapid hemolysis on blood agar, liquefied gelatin, and gave a proteolytic reaction in litmus milk. This organism was identified as a member of the genus *Pseudomonas*, and on the usual laboratory media closely resembled *P. aeruginosa* (Bergey *et al.* (1)).

Incubation Time

It was found that a seven-day incubation period (750 ml. medium, pH 7.6) was satisfactory for studying the naphthalene dissimilation when forced aeration was not used. The yield of ether extractable product (under these conditions) averaged about 0.2 to 0.3 gm. or approximately 3% by weight compared to the 7.5 gm. naphthalene added.

Effect of Aeration

The effect of forced aeration on yield was noted by passing air through a sintered glass disk as opposed to stationary growth with intermittent agitation

by hand. Eight hundred milliliters of medium (pH 7.6) was employed. Vigorous aeration yielded 1.01 gm. ether extract (12.5%) as compared with 0.33 gm. (3.7%) from the shaken culture, or a threefold increase.

Effect of pH

The data in Table I demonstrate the effect of high initial pH on the conversion of naphthalene when combined with aeration. The highest yield of ether extract obtained was 29% of the original weight of naphthalene compared to the 3% first noted. The maintenance of a higher pH during incubation may also have enabled this organism to be active for a longer period of time than when the oxidation was begun at a lower pH range.

TABLE I

EFFECT OF pH ON THE ACCUMULATION OF ETHER-SOLUBLE ACIDS DURING NAPHTHALENE OXIDATION

pH		Yield	
0 days	7 days	gm.	%
8.0	6.2	0.73	29.2
7.0	5.2	0.44	17.6
6.0	4.7	0.17	6.8
5.0	4.6	0.16	6.4
4.0	4.5	0.04	1.6

2.5 Gm. of naphthalene in 1-liter flasks, aerated.

Effect of Inorganic Salts

Several experiments on changing concentrations of inorganic salts were conducted at pH 8.0 under forced aeration in 250 ml. volumes of mineral salts. It was found that calcium carbonate (0.1–0.5%) exerted no appreciable effect. Addition of disodium hydrogen phosphate in concentrations over 0.25% exerted a deleterious effect on yield possibly because of the greater osmotic pressure. The omission of either calcium chloride or copper sulphate or both, tested after three subcultures in the deficient medium, resulted in a significant loss in yield (Table II), demonstrating that both are necessary under these conditions for the formation of good yields of product.

TABLE II

EFFECT OF CALCIUM CHLORIDE AND COPPER SULPHATE ON THE ACCUMULATION OF ETHER-SOLUBLE ACIDS DURING NAPHTHALENE OXIDATION

Medium	Final pH	Yield	
		gm.	%
No $CaCl_2$	5.8	0.35	14.0
No $CuSO_4$	6.2	0.40	16.0
No $CaCl_2$ or $CuSO_4$	5.9	0.03	1.2
Control	6.2	0.69	27.6

2.5 Gm. naphthalene in 1-liter flasks, aerated.

Isolation of Product Fractions

The crude ether-soluble product from the naphthalene dissimilation was a crystalline dark orange-colored solid with a pleasant aromatic odor. A major fraction of the crude extract was soluble in hot (55° C.) benzene from which yellow crystals precipitated on cooling. After several recrystallizations from hot benzene, a pure white product was obtained which melted at 156°–158° C. and appeared to be identical to salicylic acid (m.p. 159° C.).

The insoluble fraction (which turned brown after treatment with hot benzene) was found to char in the vicinity of 220° C.

Identification of White Crystalline Product

The white crystalline compound was identified as salicylic acid by preparation of the following derivatives: (*a*) Methylation as described by Mulliken (5) gave the typical odor of oil of wintergreen. (*b*) Nitration (5) of the unknown, known salicylic acid, and a mixture of the two all gave a derivative melting at 226°–227° C. (*c*) The amide (6) of the unknown acid and the mixture melted sharply at 139° C. Generally about 80 to 90% of the acid ether extract from the oxidation of naphthalene appeared to be salicylic acid when the oxidation was conducted under conditions favorable for products to accumulate.

Properties of Brown Fraction

The brown fraction, constituting the smaller portion of the total crude extract, was composed of several substances, none of which was identified. The fraction was colored a dark red brown (changed from orange when treated with hot benzene) and had an aromatic odor. Charring was observed to occur at approximately 250° C. after several recrystallizations from benzene. No nitrogen, sulphur, or halogens were detected. The crude material was insoluble in water, 5% sodium hydroxide, 5% sodium bicarbonate, and 85% phosphoric acid. It was soluble in concentrated sulphuric acid with darkening. Tests with ferric chloride, 2,4-dinitrophenylhydrazine, phenylhydrazine, and sodium bisulphite indicated that the fraction probably did not contain a phenol, or low molecular weight ketones or aldehydes. The mean molecular weight of the brown fraction was calculated as approximately 350.*

Discussion

It has been shown that naphthalene can be oxidized by a strain of *Pseudomonas* and that ether-soluble products may be obtained under various cultural conditions. The yield of non-naphthalenic extract calculated on the basis of 1% naphthalene in the medium was increased from 3 to 29% by adjusting the initial pH to 8.0 and aerating during incubation. The presence of calcium chloride and copper sulphate was found to be necessary for the best yields of crude product.

* *The mean molecular weight was kindly determined by Mr. George Szasz, formerly of the Department of Chemistry of The Pennsylvania State University.*

Tausson's medium (10) may have been deficient in copper which would partly account for his failure to detect any by-products. His organisms appeared to be similar to the culture used in this investigation even to the orange-brown pigments produced in the mineral salts medium.

Salicylic acid was isolated and identified as a major product of the bacterial oxidation of naphthalene under the experimental conditions described. Walker and Wiltshire (11) also isolated salicylic acid with an organism which they describe as a *Pseudomonas*. These authors found D-*trans*-1,2-dihydro-1,2-dihydroxynaphthalene as a product of bacterial as well as animal metabolism of naphthalene.

The remainder of the crude extract is probably a mixture of compounds. This fraction appeared to be free of phenol, and low molecular weight ketones or aldehydes. Solubility tests listed by Shriner and Fuson (6) indicate that quinones may be present. Since the colors of naphthoquinones range from yellow to orange and readily darken when the quinones are allowed to stand, the presence of one or more of these compounds is a possibility. The mean molecular weight of 350 for this fraction as compared to 180 for naphthalene may indicate the formation of condensation products.

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ANTIBACTERIAL ACTION OF A REACTION PRODUCT OF CYSTEINE AND IRON

III. *IN VIVO* ACTION ON PNEUMOCOCCUS INFECTION IN MICE[1]

By N. A. Hinton[2], Jack Konowalchuk[2], and G. B. Reed[3]

Abstract

A colloidal sulphur preparation, formed by autoclaving a dilute solution of cysteine and ferric ammonium citrate, was shown to have no toxicity for mice after eleven 1-mgm. intravenous doses on alternate days. Single doses up to 2 mgm. subcutaneously were not toxic but larger doses by this route produced necrosis. A single 1-mgm. dose of the preparation given to mice intravenously afforded no protection against a lethal dose of *Diplococcus pneumoniae* Type III when given simultaneously. However, groups of mice given a 1-mgm. intravenous dose of the complex and challenged with a lethal dose of pneumococcus at intervals up to 168 hr. after the therapy show no protection for the first 24 hr. following the therapy, increasing protection from 24 to 78 hr., complete protection at 78 hr., and decreasing protection from 78 to 168 hr.

As shown by Konowalchuk *et al.* (1), when cysteine hydrochloride and ferric ammonium citrate are mixed in dilute solutions and autoclaved for extended periods an insoluble precipitate is formed. This precipitated substance was initially considered to be a cysteine–iron complex, later it was demonstrated to be a form of colloidal sulphur. The substance was shown to be highly bacteriostatic *in vitro*, especially for Gram positive species of bacteria.

This paper describes *in vivo* action of the complex in pneumococcus infections in mice.

Toxicity of Sulphur Preparation for Mice

Ten mice were given 1 mgm. of the sulphur preparation, suspended in 0.5 ml. of saline, intravenously every other day for 22 days, i.e. a total of 11 mgm. The animals exhibited no signs of illness or discomfort following the injection of this milky suspension. Three weeks after the last dose they were killed by air embolism. No gross pathological changes were noted and sections of lungs, kidney, liver, and spleen gave no evidence of tissue damage.

Single doses of the complex up to 2 mgm. given subcutaneously had no local effect. Doses of 5 mgm. caused wide areas of necrosis in two to three days with extensive sloughing. Five daily doses of 8 mgm. given orally by passing a catheter into the stomach failed to produce evidence of injury.

Disposition of the Colloidal Sulphur in the Animal Body

As previously shown (1), the complex consists of rounded aggregates 1 to 2 μ in diameter which form a semistable colloidal suspension in water or physiological saline. When a dilute suspension of the complex is brought into

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Contribution from the Defence Research Board, Kingston Laboratory, Kingston, Ont.
[2] *Scientific Research Officer.*
[3] *Superintendent.*

contact with phagocytic cells, in a microscopic preparation, the particles rapidly disappear. It is assumed that they are taken up by the phagocytes. However, up to the present time no method has been found to demonstrate the particles in the cell cytoplasm. When the complex is injected into the animal, especially by intravenous or intraperitoneal routes, it appears to be taken up by reticulo-endothelial and other phagocytic cells. This is a matter of considerable interest from the point of view of chemotherapy, particularly in tuberculosis since the tubercle bacilli are known to survive for long periods in mononuclear phagocytic cells, Lurie (2), Sabin and Doan (5).

Markham and Florey (3) have shown that India ink, injected into tuberculous animals, is taken up by macrophages at the margin of older spreading lesions so that tuberculous areas were surrounded by carbon-filled mononuclear cells. In the case of younger lesions carbon-filled cells were present in the deeper layers. Markham *et al.* (4), Saunders *et al.* (6), and Su (7) found a similar wide distribution of the insoluble particulate antibiotic micrococcin. The authors discuss the probable significance of this distribution in the therapy of tuberculosis. However, the trials with micrococcin, Su (7), were disappointing, possibly owing to the very low solubility of micrococcin relative to its inhibiting potential for tubercle bacilli.

The experiments described below approach the subject indirectly by using a chemotherapeutic agent which is apparently taken up rapidly by phagocytic cells and a microorganism, the pneumococcus, which multiplies in the body largely in the extracellular fluids.

Pneumococcus Culture

When 1 ml. of a 1–100,000 dilution of a laboratory strain of *Diplococcus pneumoniae* Type III grown in heart infusion broth for 18 hr. was injected into 20-gm. white mice, the animals died in 48 hr. with little variation in the time of death. With smaller doses the results were irregular.

Simultaneous Infection and Therapy

A group of white mice of similar age and weight were given 1 ml. of 1–100,000 dilution of an 18-hr. culture of the above strain of pneumococcus intraperitoneally. Immediately after infection half the group of mice were given intravenously 1 mgm. cysteine–iron complex suspended in 0.5-ml. physiological saline, a single dose. The treated and untreated mice died in an average of 48 hr.

Advanced Therapy

A group of 120 white mice of uniform age and weight were given intravenously 1 mgm. of cysteine–iron complex suspended in 0.5 ml. of saline. The treated animals were divided into 12 lots of 10 each. The first lot of 10 was challenged, immediately after treatment, with 1 ml. of a 1–100,000 dilution of an 18-hr. culture of the above strain of pneumococcus. Other

groups were similarly challenged at intervals of 3 to 168 hr. after the single intravenous injection of cysteine–iron complex. The number of mice surviving in each group is shown in Fig. 1. It is apparent from these data that the single injection of the complex exerted no protective action in the first 24 hr. within the animal. Between 48 and 78 hr. within the animal there was a progressive increase in protective action to complete protection at 78 hr. From 78 to 168 hr. there was a progressive decline in protective action.

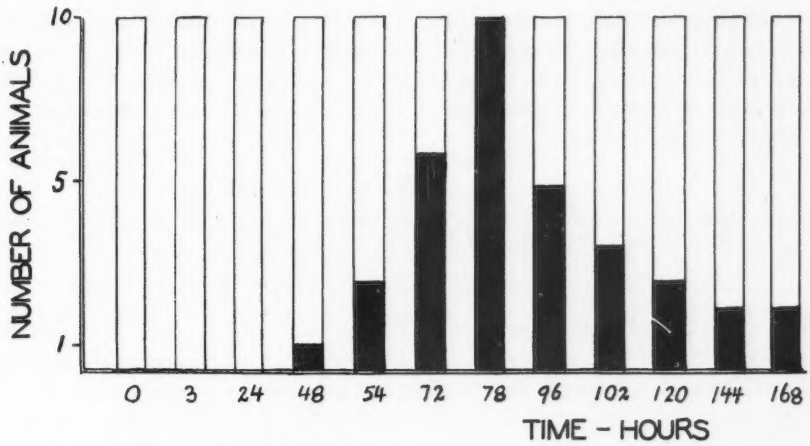

FIG. 1. Mice were given a single intravenous injection of the complex and challenged at intervals of 0 to 168 hr. later with a lethal dose of pneumococcus. The figure shows in white the number of animals which died and in black the number which survived.

Varying Doses of the Colloidal Sulphur Preparation

Similar results were obtained when the dose of the complex was varied. Groups of mice were injected intravenously with a single dose of cysteine–iron complex from 0.1 to 1 mgm., in each case suspended in 0.5 ml. of saline. At intervals of 24 to 96 hr. after the intravenous injection of complex the animals, in groups of 10, were challenged with 1 ml. of a 1–100,000 dilution of an 18-hr. culture of pneumococcus. As indicated in Table I, regardless of the size of

TABLE I

MICE GIVEN A SINGLE INTRAVENOUS INJECTION OF 0 TO 1 MGM. OF THE COMPLEX WERE CHALLENGED, IN GROUPS OF 10, WITH A LETHAL DOSE OF PNEUMOCOCCUS AT INTERVALS OF 24 TO 96 HR. AFTER THERAPY

Interval, therapy to challenge, in hours	Number of mice surviving when challenged after treatment with:				
	0 mgm.	0.1 mgm.	0.25 mgm.	0.5 mgm.	1.0 mgm.
24	0/10	0/10	0/10	0/10	0/10
48	0/10	0/10	0/10	3/10	2/10
78	0/10	3/10	4/10	8/10	10/10
96	0/10	1/10	2/10	4/10	5/10

the therapeutic dose, there was no apparent protective action at 24 hr. after therapy, slight protection with the larger doses at 48 hr., high level protection at 78 hr., and decreasing protection at 96 hr. As in the previous experiment, it is apparent that a period of approximately 78 hr. must elapse following injection of the complex before maximum protective action develops in the animals.

Daily Injections of the Complex

When daily injections of the complex were given for seven days and the animals, in groups of 10, challenged, as before, with a lethal dose of pneumococcus at intervals of 24 to 96 hr. after the last injection as shown in Table II, the highest level of protection was at 24 hr. and there was progressively less protection up to 96 hr.

TABLE II

MICE GIVEN DAILY INTRAVENOUS INJECTIONS OF 1 MGM. OF THE COMPLEX WERE CHALLENGED, IN GROUPS OF 10, WITH A LETHAL DOSE OF PNEUMOCOCCUS AT INTERVALS OF 24 TO 96 HR. AFTER THE LAST THERAPEUTIC INJECTION

Interval from last injection to challenge, in hours	No. of mice surviving after seven daily injections of:	
	0.1 mgm.	0.25 mgm.
24	5/10	8/10
48	3/10	6/10
78	3/10	5/10
96	0/10	1/10

Routes of Injection

A comparison was made of three routes of introduction of the complex. Since it was known from preliminary trials that subcutaneous and oral routes were less effective than intravenous, the dose was graded. Groups of mice were given single doses of 0.5 mgm. intravenously, 1 mgm. subcutaneously, and 10 mgm. orally. At intervals of 24 hr. to 96 hr. the mice were challenged, in groups of 10, with a lethal dose of pneumococcus. As indicated in Table III, the highest protection resulted from the small intravenous injection and, as

TABLE III

MICE GIVEN A SINGLE DOSE OF THE COMPLEX BY INTRAVENOUS, SUBCUTANEOUS, OR ORAL ROUTE WERE CHALLENGED, IN GROUPS OF 10, WITH A LETHAL DOSE OF PNEUMOCOCCUS AT INTERVALS OF 24 TO 96 HR. AFTER THERAPY

Interval from therapy to challenge, in hours	Number of mice surviving after:		
	Intravenous, 0.5 mgm.	Subcutaneous, 1 mgm.	Oral, 10 mgm.
24	0/10	0/10	0/10
48	3/10	0/10	0/10
78	8/10	4/10	0/10
96	4/10	2/10	0/10

shown in the previous experiments, the maximum protection was at 78 hr. after the therapeutic injection. Though the subcutaneous injection of twice the intravenous dose gave a much lower level of protection the maximum was again at 78 hr. after therapy. Oral therapy gave no evidence of protection even with 20 times the intravenous dose.

Discussion

The reason for the lag of some 78 hr. between the injection of the complex and the development of protection against pneumococcus infection is far from obvious. The most likely explanation is associated with phagocytosis of the injected particulate matter. If, as seems likely from the data shown in the earlier paragraphs, the complex is rapidly taken up by phagocytic cells, the pneumococcus is free to multiply in the extracellular fluids without interference by the bacteriostatic agent.

If this is correct, the complex must leave the cells in order to exert the observed delayed effect in pneumococcal infection. If the complex is liberated in particulate form it should be again taken up by phagocytic cells as in the first instance. It is suggested that the intracellular complex slowly dissolves, or is converted by metabolic activity, to an active product, and diffuses into extracellular fluid. The concentration of complex reached in the fluids will depend upon the rate of solution and diffusion of the drug as against the rate of its destruction or elimination. By this mechanism an accumulation of dissolved complex may be visualized as reaching a peak protective level in three days, after which the level declines with exhaustion of the intracellular depots of the drug.

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A CYTOLOGICAL STUDY OF THE LYSOGENIZATION OF *SHIGELLA DYSENTERIAE* WITH P1 AND P2 BACTERIOPHAGES[1]

By J. F. Whitfield[2] and R. G. E. Murray

Abstract

Cytological studies have clarified the changes within the cell during the process of lysogenization by dysentery phages P1 and P2. The first stage of infection with both phages consisted of a condensation of the nuclear chromatin into annular bodies and axial filaments; there were differences in the later stages. With P1 infections, the annular and filament stages occupied about one half the latent period. The following phase consisted of expansion of the chromatinic aggregates into complex chromatinic reticula which, in the case of lysogenization, separated into discrete normal nuclear elements. The resulting cells were indistinguishable from those of the sensitive strain. In P1 virulent infections, the same stages were observed, but the cells lysed while in the expansion phase. In P2 infections, the chromatin aggregated into axial filaments, but the filaments persisted throughout the entire latent period and persisted up to lysis in the virulent infections. During lysogenization, the filament separated into discrete normal nuclear elements. The expansion was usually more advanced at the poles of the elongate cells; the first normal cells arose from this region. There is a considerable condensation of chromatin in the infected cell but the structure of the aggregates and the mode of recovery suggest that the fundamental organization of the constituents of the nuclear organelle must be retained.

Introduction

Cytological studies of *virulent* or lytic phage infections (5, 12, 16, 17) have added to our general understanding and have provided limited, but helpful, structural information as a corollary to the detailed biochemical studies of phage infection. Little cytological information seems to be available on the effects of the *temperate* phages, which can induce in sensitive cells the potential to produce phage at some later time (13). The majority of observations have been made on the lytic cycle that may be induced in many lysogenic cells by ultraviolet irradiation and other means (6). However, Kellenberger (9) has given a brief description of the cytological effects of temperate and virulent λ phages on *Escherichia coli* K12. The process of lysogenization deserves special cytological study because it is now considered more than likely that the lysogenic property rests in a "prophage" (14) that resides in the cell nucleus, is apportioned to daughter cells with the nucleus, and is linked in some way with the genetic material of the carrier cell (3, 10, 18).

The temperate phages can elicit either a *lytic* or a *lysogenic* response when sensitive cells are infected and, usually, a rather high proportion of cells will lyse. The cells that survive are lysogenic and suffer only a transient delay in their reproduction. Thereafter, the clones behave in a normal fashion, except for occasional lysis of individual cells. Little is known of the factors that determine the relative frequencies of lytic and lysogenic responses in

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temperate infections. However, the frequencies are influenced by temperature in a system studied by Bertani and Nice (4), phage P1 acting on *Shigella dysenteriae* strain Sh; lower temperatures (down to 20° C.) favor the lysogenic response, and higher temperatures (up to 40° C.) favor the lytic response. This possibility of control was attractive for cytological studies. Phage P2, also studied by Bertani (1, 2, 3), provided another system acting on the same host with a greater amount of information on the genetical attributes, but the frequency of lysogenization is low and is not influenced by temperature. Virulent mutants of both phages occur and produce only a lytic response in the host. These systems have been subjected to cytological study and the observations will be described in this paper.

Materials and Methods

The bacterial hosts used were *Shigella dysenteriae* strain Sh, its streptomycin-resistant mutant Sh/s, and various derived lysogenic strains: Sh(P1), Sh(P2), Sh(P2 *rd l* +)(Ps + + *c*), Sh(P1)(P2), and Sh(P2 *l*). From the lysogenic strains it was possible to obtain the various temperate phages and the virulent mutants used in these studies. There was only one strain of temperate P1 and it was simply designated P1. The temperate strains of P2 were differentiated on the basis of their plaque morphology according to Bertani (1, 2). The entire phage system originated from the lysogenic strain of *Escherichia coli* known as the Lisbonne strain (11).

The medium used for all studies was tryptone broth enriched with yeast extract (LB). The composition of this medium is described by Bertani (1).

For cytological studies, the cells were first aerated in LB broth overnight in a 37° C. water bath. Aeration was obtained with an air pump. An aliquot of the overnight suspension (0.2 ml.) was placed into 20 ml. of fresh broth. The resulting suspension (0.5 ml.) was then flooded onto the surface of a well-dried Petri dish containing 30 ml. of LB agar containing 0.005 *M* calcium chloride. The plates were incubated at 37° C. for three hours at which time there were about 6×10^9 cells on the surface. Calcium chloride was added to the phage suspension (10^{10} active phage per ml.) to a final concentration of 0.01 *M* in order to ensure adsorption of the virus onto its host cell; 1.0–1.5 ml. of this suspension was flooded over the surface of the plate. This gave a phage to bacterium ratio of around 2 : 1. The phage suspension was allowed to sink into the dried agar, which took about three minutes.

Agar blocks were cut at various time intervals after infection and the cells fixed in situ in the vapor over 2.0% osmium tetroxide. Impression smears were made on cover slips, hydrolyzed in *N* hydrochloric acid at 60° C., and stained in diluted Giemsa (2 drops per ml. of *M*/15 Sörensen buffer, pH 7.0) according to the methods previously described (16, 17). Additional agar blocks were placed, cell side downward, on cover slips and fixed in Bouin's fluid (16) or methanol-formalin (15). The agar blocks were flicked off the cover slips after 45–60 min. and the fixed transfers were washed, stained in 0.01% thionin, and mounted in water.

Phase Contrast Microscopy

Three to four drops of LB agar, melted and then cooled to 45° C., were transferred to the surface of a large (43 × 70 mm.) cover slip with a Pasteur pipette and were spread evenly over an area about 2 cm. square and in a thickness about 1 mm. A block of LB agar bearing a film of Sh cells, infected using the procedure noted above, was placed, cells downward, on the puddle of agar and then quickly flicked off. This left an impression smear on the thin agar. A cover slip was then placed on the preparation and sealed with paraffin for examination with dark phase contrast optics.

Optical Apparatus

Preparations were examined with a 2 mm. apochromatic (Leitz) objective lens (N.A. 1.32), an aplanatic condenser (N.A. 1.4), a 15 × eyepiece, and Köhler illumination. For phase contrast microscopy, a Bausch and Lomb phase contrast condenser and achromatic dark phase contrast objective (N.A. 1.25) were used. Panatomic X (Kodak) cut film and, in most cases, and interference filter (5390 Å, Baird Associates) were used in photography.

Observations

The Normal Host Cell

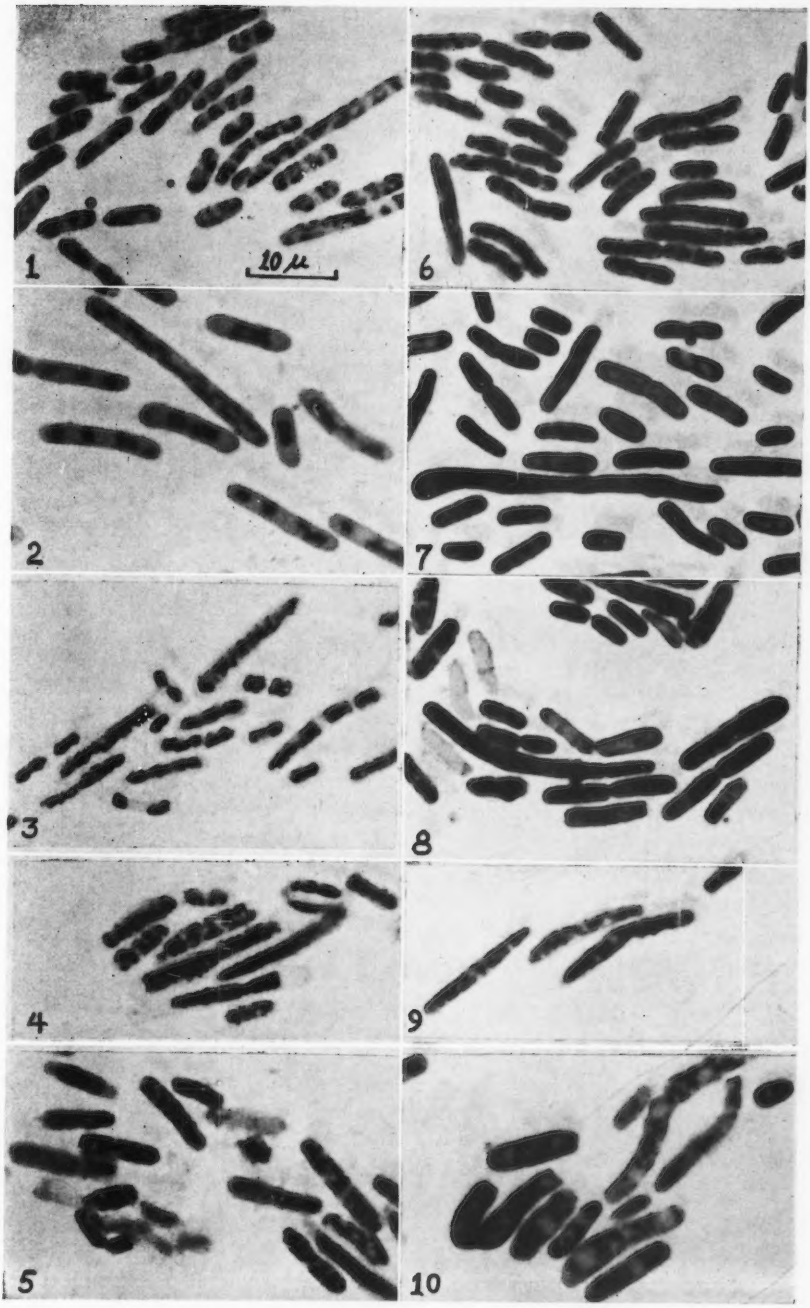
The chromatin arrangement in the normal cells of *S. dysenteriae* Sh at the stage chosen for infection is illustrated in Figs. 1 and 20. The nuclear patterns were more complex than was usually the case in *E. coli* B and *S. sonnei* (16, 17). The chromatin was dispersed in granules near the periphery of the cell and tenuous chromatic threads joined both the peripheral granules and the opposing groups. These structures were observed to be Feulgen positive and could be delineated effectively using the Azure–sulphur dioxide procedure (8).

In Bouin–thionin preparations (Figs. 6 and 24) the chromatin did not stain, as has been previous experience with many bacteria, while the cytoplasm and the areas enclosed by the chromatin were strongly basophilic. A very similar, but less contrasting, appearance was observed in the living cell using the phase microscope.

A series of photomicrographs to show the cytological events during infection with P1 phage up to the time of lysis.

FIG. 1. *S. dysenteriae* Sh. Normal cell, grown for three hours at 37° C. on LB agar. HCl–Giemsa preparation.
FIG. 2. Sh, 20 min. after infection with P1. HCl–Giemsa.
FIG. 3. Sh, 20 min. after infection with P1. HCl–Giemsa.
FIG. 4. Sh, 40 min. after infection with P1. HCl–Giemsa.
FIG. 5. Sh, 60 min. after infection with P1. HCl–Giemsa.
FIG. 6. Sh. Normal cells grown for three hours at 37° C. on LB agar. Bouin–thionin.
FIG. 7. Sh, 20 min. after infection with P1. Bouin–thionin.
FIG. 8. Sh, 40 min. after infection with P1. Bouin–thionin.
FIG. 9. Sh, 80 min. after infection with P1 vir. HCl–Giemsa.
FIG. 10. Sh, 80 min. after infection with P1 vir. Note the single cell which shows the annular structure of the nuclear aggregates. Infection of this cell was delayed. Bouin–thionin.

The micron marker on Fig. 1 applies to Figs. 1–35.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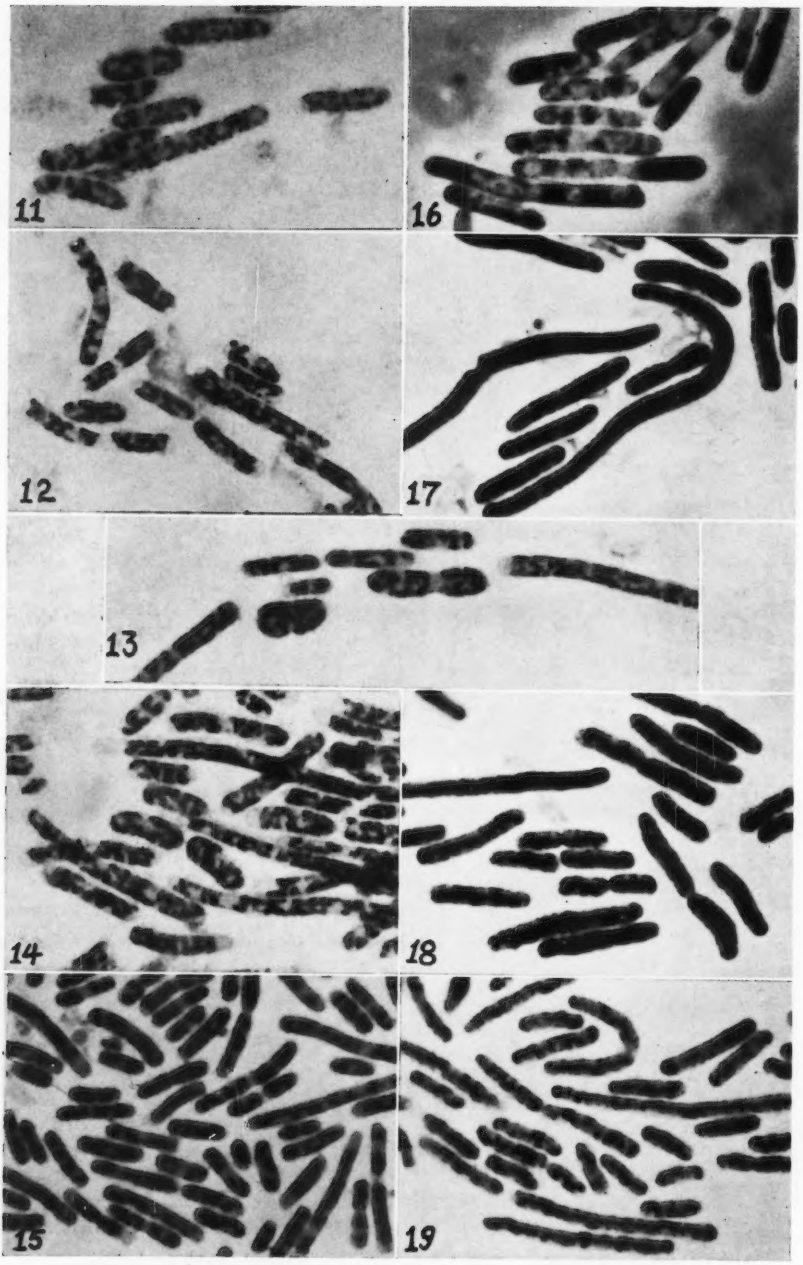
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For the purposes of this paper, a *nucleus* is defined as a group of peripheral granules or bars that are visibly interconnected by chromatinic threads. The nucleus appeared to enclose a relatively large volume of the cell and in the Bouin–thionin preparations, the enclosed portion was strongly basophilic (Fig. 24). The basophilia of both the cytoplasm and the enclosed areas was abolished by hydrolysis with *N* hydrochloric acid and by treatment with ribonuclease. The traversing chromatin threads in hydrolyzed-Giemsa preparations seemed to curve around and loculate a central portion of the nucleus. This enclosure seemed to correspond in position and properties to the achromatic "core" proposed as part of the nuclear structure of *Bacillus cereus* (15) and the term will be used for convenience.

Infections with P1 Phages

The lysis time in aerated broth cultures was approximately 40 min. and the time was virtually identical on solid medium if the experiments were done entirely at 37° C., as was the case in the phase contrast studies. However, in the cytological experiments when blocks were taken from the plates at intervals during the cycle, the time ranged up to 70 min. because of transient cooling on removal from the incubator. Lysis was practically complete using P1 virulent phage and about 85% using P1 temperate phage at 37° C. The temperate infection has been shown to be temperature sensitive and at 20° C. about 15% of the cells lyse (3, 4). The surviving cells in the temperate infections were all lysogenic and the surviving clones were cytologically indistinguishable from the sensitive strain.

The initial events in both temperate and lytic infections were identical. Five to ten minutes after adsorption of phage the peripheral nuclear granules coalesced to form central aggregates. These structures flattened and fused forming an axial filament, which was usually continuous throughout the length of the cell. Some aggregates remained independent of the fusion in the rest of the cell and retained their form. These stages are shown in Figs. 2 and 3.

The aggregates appeared to be homogeneous in osmium fixed and hydrolyzed preparations, but the use of other fixatives and the examination of living cells in phase contrast showed that there was a differentiated central region, which

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A series of photomicrographs to show stages in the recovery of normal morphology and growth following infection with P1 phage. All except Fig. 16 from same experiment as Figs. 1 and 3–8 on Plate I.

FIG. 11. Sh, 90 min. after infection with P1. HCl–Giemsa.
FIG. 12. Sh, 120 min. after infection with P1. HCl–Giemsa.
FIG. 13. Sh, 150 min. after infection with P1. HCl–Giemsa.
FIG. 14. Sh, 150 min. after infection with P1. HCl–Giemsa.
FIG. 15. Sh, 180 min. after infection with P1. HCl–Giemsa.
FIG. 16. Sh/s, 40 min. after infection with P1. Note the annular structure of the nuclear aggregates. Methanol-formalin-phase contrast.
FIG. 17. Sh, 90 min. after infection with P1. Bouin–thionin.
FIG. 18. Sh, 120 min. after infection with P1. Bouin–thionin.
FIG. 19. Sh, 180 min. after infection with P1. Bouin–thionin.

was surrounded by the chromatin. This differentiation is shown in individual cells in Figs. 10 and 16. Because of this structural form the aggregates have been termed "annuli" to follow the terminology of Kellenberger (9). We believe that the central portion represents a relict of the core of the normal nucleus. The axial filament, like the annuli, could be shown, in many instances, to have an achromatic central region.

The process of axial filament formation was complete by the mid-point of the lysis time and this proportion was maintained whatever the lysis time in the particular experiment, and thus ranged from 20 to 35 min. These observations, on hydrolyzed-Giemsa or equivalent preparations, were checked by observations on living cells using phase microscopy. In Bouin–thionin (Fig. 7) or MF–thionin preparations the annuli and axial filament stood out as unstained areas in the midst of an uniformly stained cytoplasm and the changes during the early stages of infection reflected those shown by the hydrochloric acid–Giemsa technique. During this time the cytoplasmic basophilia was virtually unchanged.

At the mid-point of the lysis time, i.e., 20 min. in the experiments truly comparable to those of Bertani (4), the axial filament started to expand (Fig. 3). The continuous chromatin rim of the filament fragmented into a number of separate granules and bars, which moved peripherally to form a complex chromatin reticulum (Figs. 3 and 4). In the hydrolyzed preparations this network appeared to be vacuolated, but these areas were stained directly with thionin (Figs. 4, 5, and 8) and this basophilia was abolished by treatment with *N* hydrochloric acid and with ribonuclease. It was felt that this material represented the fragmentation and development of the core. During this process of expansion the width of the cells increased but there was no evident increase in length.

It was at this stage of events that a proportion of the cells started lysis in the temperate infections and all the cells lysed in the virulent infections. The process and the time of lysis was identical in each case. The cells that were to lyse were not particularly distinguished from lysogenizing cells at the same stage except that the chromatin appeared to be more finely stippled and granular. The identity of these cell types was checked by lowering the temperature during the first 20 min. of the latent period, thus increasing the proportion of lysogenizing cells. Soon before lysis there was a rapid swelling of the cell, with the appearance of large vacuoles, which were particularly

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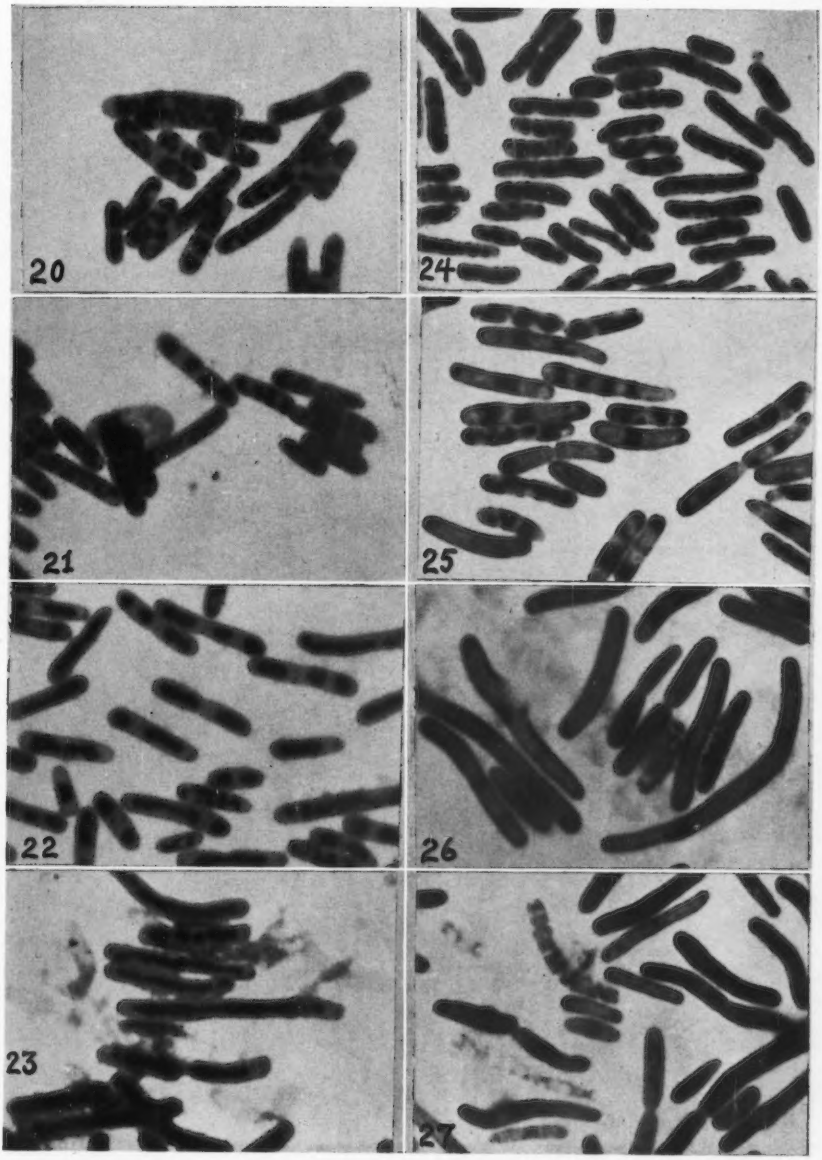
A series of photomicrographs to show the cytological events during infection with P2 virulent phage. All taken from one experiment.

FIG. 20. Sh. Normal cells grown for three hours at 37° C. on LB agar. HCl–Giemsa.
FIG. 21. Sh, 20 min. after infection with P2 (virulent). HCl–Giemsa.
FIG. 22. Sh, same experiment as Fig. 21, 30 min. after infection. HCl–Giemsa.
FIG. 23. Sh, same experiment ast Fig. 21, 40 min. after infection. HCl–Giemsa.
FIG. 24. Sh, normal cells grown for three hours at 37° C. on LB agar. Bouin–thionin.
FIG. 25. Sh, 30 min. after infection with P2 (virulent). Bouin–thionin.
FIG. 26. Sh, same experiment as Fig. 25, 40 min. after infection. Bouin–thionin.
FIG. 27. Sh, same experiment as Fig. 25, 40 min. after infection. Bouin–thionin.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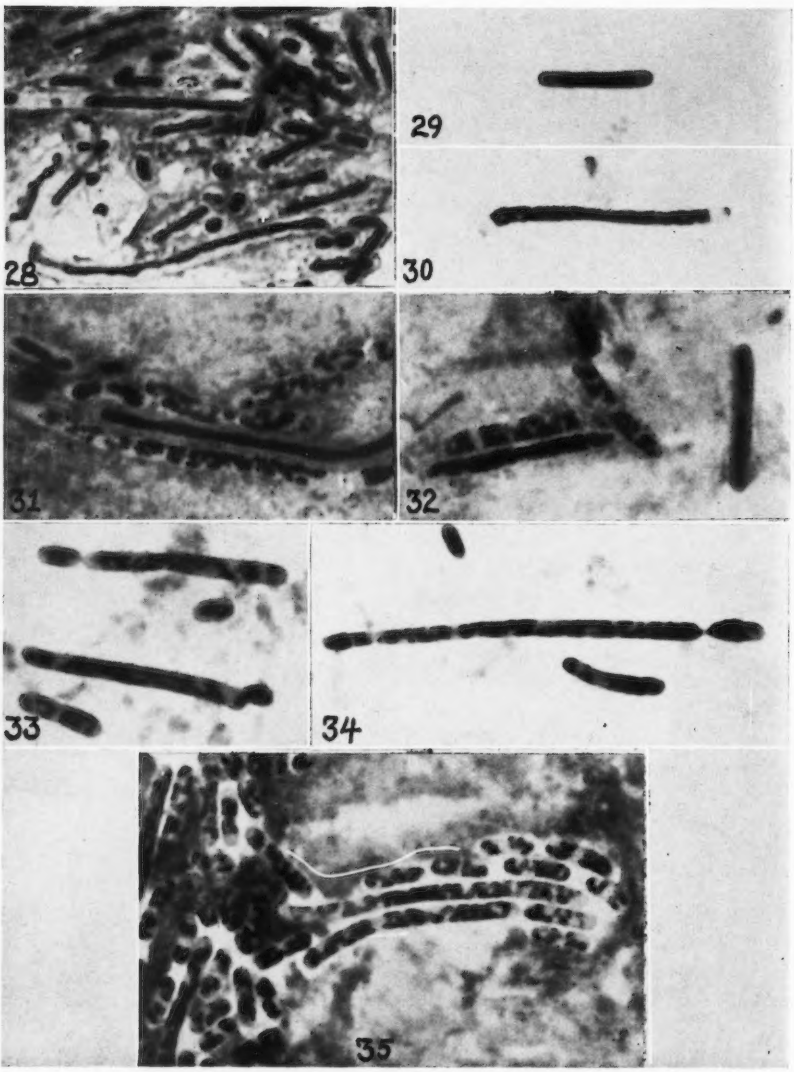
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prominent in the Bouin–thionin preparations (Figs. 9 and 10). In phase contrast studies of the living process the impression was given of a spongework or mesh within the cell. During the terminal swelling, bright "bubbles" with dense borders appeared in the spongework. As lysis approached there was a loss of the appearance of granulation, the cell decreased in density (became brighter), swelled, and disintegrated quite suddenly.

In the temperate infections the cells with coarsely reticulated chromatin and greater than normal width remained after this partial lysis. There followed a further increase in size; first, in width (Fig. 11) and, later, in length with a concomitant decrease in width (Figs. 12, 13, and 17). At 120 to 150 min. after infection the cells were very filamentous and there was the beginning of division of the filaments (Figs. 12, 13, 14, 17, and 18). By 180 min. the segmentation of the filaments, now of nearly normal width, was almost complete and the chromatin pattern of the cell had returned to normal (Figs. 15 and 19). In several of these experiments it was shown that the surviving cells were lysogenic and also that there was no cytological distinction between the sensitive and the lysogenic strains of Sh.

The lysogenic cells Sh(P1) still adsorbed both the temperate and the virulent P1 phages. Superinfection with the virulent phage leads to lysis and the cytological events appeared to be identical to those described. Superinfection with the temperate phage does not lead to any apparent disturbance of the growth rate of the culture. However, cytological preparations showed that the chromatin aggregated into annuli and axial filaments within 10 min. of infection. The axial filaments expanded very rapidly and the chromatin configurations became indistinguishable from the normal *within 30 min.* of the adsorption of phage.

Infections with P2 Phages

The initial cytological events were similar to the infections with P1 phages. The chromatin of each nucleus coalesced to form annular structures 5 to 10 min. after infection, and then these fused into an axial filament (Figs. 21, 22, 25, and 26). However, the axial filament remained intact until shortly before lysis (Figs. 23 and 27), which under ideal conditions occurred 37 to 40 min. after infection. Unlike P1 infected cells, those cells infected with either virulent or temperate P2 phages increased rapidly in length but not in width,

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A series of photomicrographs to show stages in the recovery of normal morphology and growth following infection with P2 *rd l* + phage. These photomicrographs are not all from the same experiment but are all stained with the HCl–Giemsa procedure.

FIG. 28. Sh/s, 45 min. after infection.
FIG. 29. Sh/s, 90 min. after infection.
FIG. 30. Sh/s, 100 min. after infection.
FIG. 31. Sh/s, 90 min. after infection.
FIG. 32. Sh/s, 110 min. after infection.
FIG. 33. Sh/s, 150 min. after infection.
FIG. 34. Sh/s, 225 min. after infection.
FIG. 35. Sh/s, 230 min. after infection.

As lysis approached, the cytoplasmic basophilia became less intense (Figs. 26 and 27); the axial filament became fragmented and was invaded by areas of basophilia with an intensity of staining equal to that of the cytoplasm. In the living cell (phase contrast), the homogeneously bright filament became less distinct as lysis approached and assumed a granular aspect. This was probably an *abortive* expansion phase reminiscent of the earlier and more complete expansion phase in P1 infections. However, the expansion was not very marked, the parts of the filament remaining relatively compact.

During the latter part of the latent period, the only area in which chromatin was found was in the axial filament. This is very different from other phage–host systems such as T5–*E. coli* B (17) and T2–*E. coli* B (12, 16) where there is a widely distributed synthesis of new chromatin throughout the cell during the latent period.

The proportion of cells lysing in infections with the various temperate mutants of P2 varied considerably. P2 *rd l* caused lysis in about 85% whereas P2 *l* caused lysis in about 99% of infected cells. For this reason, the salient features of the lysogenization process were studied using P2 *rd l*. The changes observed with this phage were the same with P2 *l*.

As recovery commenced, the 'core' of the axial filament appeared to expand and the chromatinic periphery migrated towards the cell boundary. (Figs. 29, 30, 31, and 32). The chromatin segmented into a double row of thick bars and granules. The solid bars then assumed a beaded aspect and subdivided into smaller granules interconnected by threads of chromatin (Figs. 33 and 34). The 'core' generally did not expand uniformly along the length of the cell. The expanding filaments assumed a 'looped' appearance because the process of expansion in the middle of the cell lagged behind that in the polar regions (Fig. 30).

From living, infected cells under the phase contrast microscope the relation between expansion and the resumption of cytoplasmic growth was found. About 50 min. after infection (10 min. after lysis) the poles of the lysogenizing cell appeared to increase greatly in density, throwing the axial filament into bold relief. The cell then began to increase in length while the filament remained unchanged. Expansion of the axial filament, which immediately followed this stage, was heralded by the appearance within the optically homogeneous filament of darker areas giving the filament a spotted appearance.

The final stages of lysogenization were made up of two steps which overlapped one another (Figs. 31, 32, 33, and 34). The first step was a reduction of the heavy chromatinic peripheral bars into smaller granules. The second step was a separation of groups of granules and threads during movement to the periphery of the cell. When one or two separate nuclear elements had been formed, the part of the cytoplasm in which they were embedded was cut off and together with the nuclei made up a normal but lysogenic cell (Fig. 35).

A synchronous transition from stage to stage was virtually impossible to attain on agar. Hence, with stained preparations a wide range of time

intervals existed for each phase. However, with phase contrast, it was possible to study single cells and hence obtain accurate time intervals. The complete cycle from infection to the production of the first normal appearing but lysogenic cell took approximately 90 min. When the *whole population* had to be taken into account, the cycle required about 180 min.

Discussion

The structure of the normal cell and the changes during infection are summarized in schematic form in Fig. 36. The nuclear equivalents of the normal Sh cell during the first hour of exponential growth seem to consist of chromatin granules connected by chromatin threads forming a reticulum which enmeshes and surrounds an achromatic "core". We believe that the core contains ribonucleic acid (RNA) as a major component because the basophilia of this area is abolished by both *N* hydrochloric acid and ribonuclease treatment. The initial event in infection with the P1 and P2 phages

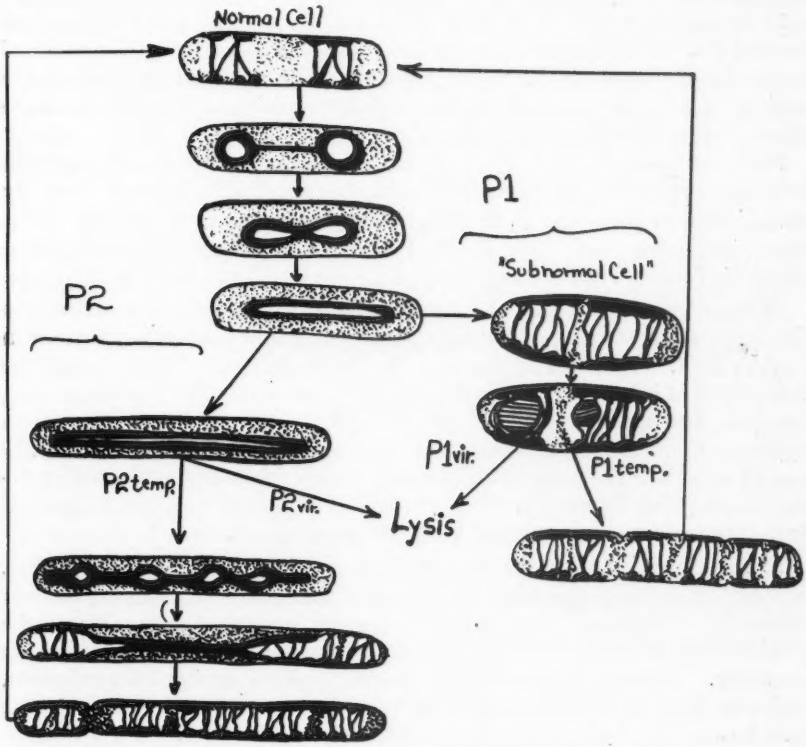

FIG. 36. Diagrammatic representation of the cytological effects of P1 and P2 phages on Sh or Sh/s.

involves the condensation of the chromatin around the core so that each separate nuclear element appears to be an annulus of chromatin. The annuli within a single infected cell pile up in the center of the cell forming an axial filament, which retains the basic structure of the nucleus, i.e. a border of chromatin surrounding the remains of the core. The axial filament stage occupies about one half of the latent period in P1 infections; in P2 infections, the axial filament is retained up to lysis. The condensation of nuclei into an axial filament seems to depend on the degree of separation of the nuclear elements. Many dividing and divided nuclei in normal cells show chromatinic connecting strands; these connections may govern the fusions.

Bertani and Nice (4) found that the proportion of lysogenesis in P1 infections was increased by cooling during the first half of the latent period. Thus, the time of the "decision" for lysis or lysogenesis could be defined and the critical change occurred at about 20 min. after infection. This critical time is coincident with the expansion of the axial filament. After the lytic decision has been made the cell will presumably be forced to divert most of its synthetic mechanisms to the construction of new phage. The host desoxyribosenucleic acid (DNA) may be eliminated as in T2 infections phage DNA synthesized instead (7). It seems likely that phage DNA in P1 infections is synthesized along the elements of the expanding chromatin reticulum in view of the similar appearance of both lysing and lysogenizing cells. However, the observations do not allow appraisal of these possibilities.

For lysogenesis, the basic chemical and morphological entities must remain intact, otherwise it would be unlikely to have cytologically and physiologically normal cells as the result of infection. In lysogenizing cells, the host DNA must also increase and, cytologically, there is a visible increase in the nuclear material of the cell, which eventually subdivides into a number of normal cells.

Prior to lysis in both P1 and P2 infections, there is a noticeable decrease in the intensity of staining of the cytoplasm with thionin following fixation in Bouin's fluid. This may be due to a drop in the content of RNA during the late phases of infection but, equally, could be due to dilution in an expanding system. The synthesis of cytoplasm in recovering cells in the P1 system appears to lag behind the resumption of chromatin synthesis. However, in the P2 infections the resumption of cytoplasmic growth begins as soon as the expansion phase is started. The nucleus in this case re-forms its normal formations within the bounds of a steadily growing cell.

In superinfection with the temperate phage (i.e. Sh(P1) exposed to P1), no development of phage takes place, axial filaments form but quickly recover without the formation of the greatly expanded subnormal cells. Hence the production of axial filaments is a consequence of adsorption and not of phage synthesis. This indicates that the recovery phase during lysogenization, which is much more prolonged, must have some special characteristics that may be associated with the development of the carrier state.

Whitfield (unpublished) has studied the phenomenon of axial filament production by changing the environmental condition of normal Sh cells. The

results seem to indicate that filaments are produced by the action of cations on the anionic DNA of the cell. Free cations (Na^+ and K^+) would accumulate within the cell as a result of the diminution of cellular metabolism or direct membrane effects of phage adsorption on the cell's surface. If this is true, the production of axial filaments may be regarded as a purely secondary result of a primary metabolic effect of the phage on its host. It is not yet possible to determine if this phenomenon has any significance in the active production of new phage or the process of lysogenization. The effect achieved is the compression of the individual units of the nucleus into a tightly packed mosaic.

Kellenberger (9) infected *E. coli* K12S and K12 (λ) with λ virulent and λ temperate phages. After infection with the virulent phage the nuclei became aggregated ("les nucléoïdes . . . sont comme empâtés"). As time progressed, the nuclear structures became more diffuse, i.e. fragmented. Cells about to lyse were uniformly granular. The initial cytological changes imposed by the temperate phage on the host were the same as those observed in infections with the virulent phage. However, the cells which were to become lysogenic did not pass through the diffuse granular stage. A similar situation is found in the systems under discussion. The temperate and virulent phages share a common sequence of early effects.

Virulent phages such as T2 (12, 16) and T5 (17) destroy the identity of the nuclear sites. However, the P phages do not appear to disrupt the basic organization of the host's nuclei and this property certainly should facilitate lysogenization.

Acknowledgments

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A REVISION OF HOLMES'S CLASSIFICATION OF ANIMAL VIRUSES, SUBORDER III (ZOOPHAGINEAE)[1]

By C. E. van Rooyen

Abstract

Holmes's classification of the animal viruses, Suborder III, Zoophagineae, has been revised and extended. Four new families, eight new genera, thirty-one new species, sixteen new combinations, and nine new subspecies have been added. A list of these is contained in the summary of proposals. The International Bacteriological Code of Nomenclature provides guidance for the use of the Linnaean system of binomial nomenclature in the orderly naming of a wide range of microbial species. Like the bacteria, the viruses are amenable to orderly arrangement and classification, and the several types may be satisfactorily named.

Introduction

The first comprehensive proposals for the taxonomy and nomenclature of the Order Virales was published by Holmes (74) and embraced viruses pathogenic to bacteria, plants, and animals with following suborders: Suborder I, Phagineae; Suborder II, Phytophagineae; and Suborder III, Zoophagineae. Holmes's original classification has been revised by several workers, notably Sompolinsky (148), Steinhaus (149), Merchant (114), and Ansel (4).

Independent proposals for the classification of human and animal viruses and the rickettsia have recently been published by Zhdanov (172) in 1953. Zhdanov (172) has approached the classification of viruses from a different angle to that adopted by Holmes (74). All the organisms included in the Order Rickettsiales in the sixth edition of Bergey's Manual are grouped with the human and animal viruses of the Order *Virales* under the phylum *Vira*. Thus the rickettsias proper, the bartonellas, and the chlamydozoa are grouped together with the filterable viruses.

Zhdanov's monograph cites references to the classification of filterable viruses by W. L. Ryshkov. The latter has grouped viruses according to the structure of the virus particles and has integrated the plant viruses with the human and animal viruses.

To sum up, it may be said that the two systems of classification of viruses are basically quite different although both include the species described in the sixth edition of Bergey's Manual plus additional named species. Since Zhdanov (172) does not recognize names employed by Holmes (74), this classification would appear to be an alternative for Holmes' (74) classification rather than a revision or an extension of Holmes' work, within the rules of the International Code (23). Under the circumstances, it is difficult, if not even impossible, to revise Zhdanov's (172) classification in accordance with the Code.

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Contribution from the University School of Hygiene, Connaught Medical Research Laboratories, and the Department of Bacteriology, University of Toronto, Toronto, Ontario.

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In conformity with the procedure followed by Holmes (74) and others, the present revision has accorded major consideration to symptomatology, host specificity, and cellular affinity as basic criteria for the formation of families, genera, and species. Criteria such as these are broad enough to provide a basis for proceeding with the task of classifying viruses according to the diseases that they produce by use of the existing rules of taxonomy.

It should be remembered that the International Bacteriological Code of Nomenclature, Section 5, Rule 17, makes adequate provision for any future legitimate changes in names as a result of segregation or union or groups or change in the rank of groups. Likewise, Chapter 4 contains provisions for exceptions to the rules and for the interpretation and modification of rules. Thus it may be said that the International Bacteriological Code of Nomenclature not only provides the necessary mechanism for the naming of viruses, but also contains the authority whereby future alterations and amendments may be effected following orthodox taxonomic practices. (See Section 6, Rules 23, 25.)

Viruses occupy a unique position in the realm of microbiology. Outside the susceptible cell, they behave as inanimate objects which are apparently devoid of the ability to reproduce and lack demonstrable physiological activity. It is only after entry of a virus into the cells of a susceptible host that the characteristics of the virus become manifest. In short, animal tissue provides the only medium through which many viruses are able to reveal their characteristics.

In attempting to group animal viruses one must appreciate that, whereas abundant data are available regarding the properties of some, only relatively little is known of others. But this same situation is also applicable to the bacteria, and, for instance our failure to cultivate the leprosy bacillus or to fulfill Koch's postulates in the case of some others has not delayed the inception of a general classification for the bacteria as a whole. A somewhat similar situation is applicable to the Fungi Imperfecti which are divided into families, genera, and species. Thus, it is advisable to adhere to a well tried system of taxonomy which has withstood the test of time and is sufficiently flexible to embrace microbial agents as a whole, rather than to limit the classification of viruses to a few groups which have been completely studied. For reasons such as this, attempts to form groups or genera on a piecemeal basis would be doomed to failure.

Andrewes (3) has proposed that viruses should be classified according to morphology, method of reproduction, chemical composition, physical properties, susceptibility to chemical agents, and other data. At first sight it would appear to be relatively easy to evolve an excellent classification of viruses employing such criteria. Closer examination of the situation, however, reveals that the range and choice of criteria presently available do not lend themselves to the formation of family groups or generic relationships. Neither is it possible to weld together a multiplicity of heterogeneous characters to form a workable and practical classification. For instance, the size and morphology of all the viruses is not known. Some have been measured by ultrafiltration alone, and others by electron microscopy.

A few viruses seem to possess distinctive shapes. The variola–vaccinia group resemble rectangular shaped structures possessing a central area of electronic density. The viruses of influenza and avian Newcastle disease display variable appearances which, unfortunately, do not lend themselves to scientific description. It is also possible that some of the differences in structure noted may be induced by the process of drying under high vacuum and may represent artefacts. One should also look to the future in regard to the growing evidence that certain viruses may occur in soluble phase. For this reason too much emphasis should not be placed on morphology as a criterion for classification. The viruses of infectious hepatitis and serum jaundice have not yet been seen or cultivated, and notwithstanding the ease with which the diseases they cause are identified by clinicians throughout the world, their associated viruses could not be named or classified.

To extend such criteria to fit the broad pattern of a working classification is clearly impossible at present. The same remarks can be applied to hemagglutination as a potential characteristic and basis for classification. The phenomenon of hemagglutination as applied to the study of virus infection and its possible employment in the field of taxonomy is well explained by Hirst (73) in the following sentences:

"There are 11 species of virus known to agglutinate red cells: pneumotropic viruses, influenza (human, swine), PVM (mouse pneumonia), and strain 1233 (human; dermotropic viruses, variola, vaccinia and ectromelia (pox virus of mice); neurotropic viruses, Theiler's virus (enteric and encephalitic disease of mice) encephalomyocarditis virus (endemic rat disease); pantropic viruses, fowl plague and Newcastle disease (generalized infections of fowl) and a virus with glandular tropism, mumps. It is obvious that this is a very heterogeneous group of agents in respect to the species of natural host, organ specificity, pathogenicity, and virus particle size. The latter varies from very small to very large. Each virus has its own spectrum of red cell species with which it can combine. In a few cases, the spectrum is narrow and largely confined to red cells from infectible species."

Efforts to classify viruses according to differences in chemical composition are equally unsatisfactory for several reasons. For example it would be necessary to obtain relatively pure suspensions of every known virus in sufficient quantity and purity to enable chemical analysis to be undertaken. It is also possible that many of the earlier biochemical studies may be reinvestigated with the aid of radioactive tracer techniques.

The bacteriologist has grown accustomed to identifying bacteria by the performance of *in vitro* tests, not infrequently without regard to the source of origin of the material examined and usually without recourse to animal inoculation tests. The method of procedure employed by the virologist is different. He is compelled to know the source of origin of the diseased tissue or starting material for without the employment of animal inoculation and serum neutralization tests, he cannot proceed. The introduction of tissue cultivation techniques, synthetic media, and single cell pure lines of tissues

may still further reveal the complexity of virus host cell relationships. It is relatively easy for the bacteriologist to group organisms into taxa comprising a maximum of homogenicity of morphological and physiological characters, but the same is virtually impossible to accomplish at the present time in the case of order *Virales*.

Perhaps, ultimately, greater use may be made of such criteria as morphology, hemagglutination, and chemical composition. Their inclusion in the writer's opinion must necessarily be confined to supplementing and reinforcing the more fundamental basis for classification of viruses as proposed by Holmes, namely that founded on clinicopathological features and species susceptibility.

Whilst these academic considerations will be subject to endless debate, many viruses and the maladies which they produce, such as rabies, poliomyelitis, measles, chicken pox, variola, vaccinia, mumps, yellow fever, infectious hepatitis, serum jaundice, and others will continue to remain stable relationships for all time. For centuries past, man has recognized virus diseases from their clinical symptomatology and pathological lesions. There is every indication this will remain so and prove to be as permanent characters as others encountered in the realm of microbiology.

Proposals

The revised arrangement now proposed, comprises the creation of four new families, eight new genera, thirty-one new species and sixteen new combinations, and nine new subspecies.

ORDER VIRALES—Breed, Murray, and Hitchens

See Holmes (74)

Suborders

I. Infecting bacteria. *Phagineae* Holmes (74).

II. Infecting plants. *Phytophagineae* Holmes (74).

III. Infecting animals. *Zoophagineae* Holmes (74).

Revised Key to the Families of Suborder III, **Zoophagineae** Holmes (74)

I. Viruses causing diseases of insects only. *Borrelinaceae* Holmes (74).

II. Viruses of the human and animal pox group. *Borreliotaceae* Holmes (74).

III. Viruses causing proliferative tissue lesions in man and animals. *Molitoraceae* fam. nov.

IV. Viruses of the encephalitis group. *Erronaceae* Holmes (74).

V. Viruses isolated from insects in the forests of Africa and South America by intracerebral inoculation of mice. *Smithburniaceae* fam. nov.

VI. Viruses recovered from feces of human cases of herpangina and pleurodynia. *Dalldorfiaceae* fam. nov.

VII. Viruses causing generalized systemic infection in man and animals. *Reedellaceae* fam. nov.

VIII. Viruses of the infectious anemia group. *Trifuraceae* Holmes (74).

IX. Viruses exhibiting special affinity for salivary gland tissue. *Rabulaceae* Holmes (74).

ORDER VIRALES—Holmes (74)

SUBORDER III. **Zoophagineae** Holmes (74)

FAMILY II. BORRELIOTACEAE HOLMES (74)

Viruses of the pox group, inducing diseases characterized in general by discrete primary and secondary lesions of the nature of macules, papules, vesicles, or pustules. Size range by electron microscopy, 322–10 mμ. Circular or rectangular in shape, some with a central area of electronic density. Transmissible by direct contact or droplet infection.

Key to the genera of family **Borreliotaceae**

I. Viruses of the animal pox group.
Genus I. *Borreliota* Holmes (74).

II. Viruses of the human pox group.
Genus II. *Briareus* Holmes (74).

III. Viruses of the human measles group.
Genus III. *Morbillifex* gen. nov.

IV. Viruses of the animal foot and mouth disease group.
Genus IV. *Hostis* Holmes (74).

V. Viruses of the herpes group affecting man and animals.
Genus V. *Scelus* Holmes (74).

Genus I. **Borreliota** Goodpasture (61)

Viruses of the animal Pox Disease Group, inducing diseases characterized by formation of papules, pustules, and scabs, shed with or without scarring. Genus named after *Borrel*, investigator who first discovered the specific elementary bodies of fowl pox with the Latinized name of the smallest Greek letter, *iota*, signifying the smallest particle. The name *Cytoryctes variolae* Guarnieri 1892 was based on intracellular inclusions, Guarnieri bodies, as supposed sporozoan parasites, Calkins (26).

The type species is *Borreliota avium* Goodpasture.

Key to the species of genus **Borreliota**

I. Affecting the domestic fowl, turkey, pigeon, and canary.
1. *Borreliota avium* Homes (74). (Fowl pox virus).
2. *Borreliota meleagridis* sp. nov. (Turkey pox virus). Beaudette and Hudson (9).
3. *Borreliota fringillae* sp. nov. (Canary pox virus). Kikuth (88); Kikuth and Gollub (89); Burnet and Barnard (24); Burnet and Lush (25); Reis and Nobrega (124); Herzberg (71).
4. *Borreliota columbae* sp. nov. (Pigeon pox virus). Juliusberg (80).

II. Affecting the cow.
5. *Borreliota jenneri* sp. nov. (Cow pox virus). Jenner (79); Reece (122); Downie (46); Davies, Janes and Downie (38).

III. Affecting the rabbit.
6. *Borreliota cuniculi* sp. nov. (Rabbit pox virus). Greene (62, 63, 64, 65); Rosahn and Hu (127); Rosahn, Hu, and Pearce (128).
7. *Borreliota myxomatis* comb. nov. (Myxoma virus of rabbits). *Molitor myxomae* Holmes (74).

IV. Affecting the sheep.
8. *Borreliota ovina* comb. nov. (Sheep pox virus). *Variola ovina*. Blaxall (17); Borrel (20); Bennett, Horgan and Haseeb (14).

V. Affecting swine.
9. *Borreliota suis* Holmes (74). (Swine pox virus). *Variola suilla*. Blaxall (17).

VI. Affecting the mouse.
10. *Borreliota marmorans* comb. nov. Holmes (74). (Virus of infectious extromelia or mouse pox). *Scelus marmorans* Holmes (74).

Specific epithets for the following species have been proposed by Blaxall (17), namely, *Variola caprina* (Goat pox virus) used by Blanc (16), and *Variola equina* (Horse pox virus). It is unlikely that goat and horse pox, respectively, constitute specific virus entities.

Genus II. **Briareus**

Viruses causing disease of the Varicella, Variola, Alastrim, and Zoster Group, characterized by reddened spots and rings in affected tissues, becoming papular or vesicular. Associated with the formation of elementary and intracellular inclusion bodies. Generic name from Latin *Briareus*, name of a hundred-armed giant.

The type species is *Briareus varicellae* Holmes (74).

Key to the species of genus **Briareus**

I. Causing chicken pox in man.
1. *Briareus varicellae* Holmes (74).

II. Causing variola or smallpox in man.
2. *Briareus variolae* comb. nov. Holmes (74). *Borreliota variolae* Holmes (74).

III. Causing alastrim or kaffir pox in man.
3. *Briareus torresii* sp. nov. Torres and Teixeira (163); da Cunha and Teixeira (32); Torres (162); Horgan and Haseeb (75); Horgan *et al.* (76).

IV. Causing Zoster or shingles in man.
4. *Briareus zosticus* sp. nov. Kundratitz (97); Lipschutz and Kundratitz (101); Siegl (139); Netter (116); Cleveland (30); Bruusgaard (21); Paschen (118).

Genus III. **Morbillifex**

Viruses of the human measles group. Name from the Latin *morbilli*, measles, and *facio*, to make or produce.

The type species is *Morbillifex morbillorum* comb. nov.

Key to the species of genus **Morbillifex**

I. Causing classical measles in man.
1. *Morbillifex morbillorum* comb. nov., *syn. Briareus morbillorum* Holmes (74).

II. Causing rubella or German measles in man.
2. *Morbillifex embryorum* sp. nov. Gregg (66); Swan (151); Swan *et al.* (152, 153); Evans (47); (48); Reese (123); Krause (94); Long and Danielson (102); Winterbotham (170).

III. Causing roseola subitum or sixth disease in man.
3. *Morbillifex subitus* sp. nov. Zahorsky (171); Clemens (29); Cushing (34); Cutts (35).

Genus IV. **Hostis**

Viruses of the Foot-and-Mouth Disease Group, inducing diseases mainly characterized by vesicular lesions. Primarily affecting cattle, sheep, horses, and goats, causing vesicular eruptions on the mucous membranes of the lips, gums, and tongue. Generic name from Latin *hostis*, enemy or stranger.

The type species is *Hostis pecoris* Holmes (74).

Key to the species of genus **Hostis**

I. Affecting cattle and other animals with cloven hoofs; horse immune or highly resistant.
1. *Hostis pecoris* Holmes (74). (Foot-and-Mouth disease in virus).

II. Affecting horses.
2. *Hostis equinus* Holmes (74). (Vesicular stomatitis virus of horses, cattle, and swine).

III. Affecting swine.
3. *Hostis exanthematis* Packer, see Merchant (114). (Vesicular exanthema of swine virus).

IV. Affecting sheep.
4. *Hostis ecthymatis* Packer comb. nov. *Borreliota ecthymatis* Packer, see Merchant (114). (Contagious ecthyma virus of sheep).

Genus V. **Scelus**

Viruses of the Herpes Group, inducing diseases characterized in general by vesicular primary lesions, sometimes with subsequent involvement of the nervous system. Generic name the Latin neuter noun *scelus*, rascal.

The type species is *Scelus recurrens* Holmes (74).

Key to the species of genus **Scelus**

I. Cause of so-called fever blisters in man, herpes febrilis.
1. *Scelus recurrens* Holmes (74).

II. Cause of virus *B* infection in monkey, transmissible to man and causing rapidly fatal ascending myelitis.
2. *Scelus beta* Holmes (74).

III. So-called virus III of rabbits. Course of the induced disease in nature unknown.
3. *Scelus tertium* Holmes (74).

IV. Cause of ovine balanoposthitis in sheep.
4. *Scelus ulceris* Holmes (74).

V. Cause of erosive stomatitis in cattle.
5. *Scelus bovinum* Holmes (74).

FAMILY III. MOLITORACEAE Fam. nov.

(See Family II, Genus V. Holmes (74))

Viruses causing proliferative lesions in man or animals, some of which may spontaneously or artificially be induced to undergo malignant change. Sometimes associated with the presence of inclusion and elementary bodies. Viruses exhibiting a high degree of host specificity. Some with long incubation period. Spread by direct contact. Generic name from Latin *molitor*, contriver.

The type species is *Molitor verrucae* Holmes (74). Single genus **Molitor.**

Key to the species of genus **Molitor**

I. Affecting man.
1. *Molitor verrucae* Holmes (74). (Human wart virus, skin and venereal).
2. *Molitor hominis* Holmes (74). (Molluscum contagiosum virus).

II. Affecting the cow.
3. *Molitor bovis* Holmes (74). (Cattle wart virus).

III. Affecting the dog.
4. *Molitor buccalis* Holmes (74). (Dog lip wart virus).

IV. Affecting the rabbit.
5. *Molitor gingivalis* Holmes (74). (Rabbit oral papillomatosis virus).
6. *Molitor sylvilagi* Holmes (74). (Shope papilloma virus).
7. *Molitor fibromatis* sp. nov. Shope (137, 138); Berry and Dedrick (15); Hurst (78). (Shope fibroma virus).

V. Affecting the chicken.
8. *Molitor tumoris* Holmes (74). (Rous sarcoma virus).

VI. Affecting the frog.
9. *Molitor luckei* sp. nov. (Adenocarcinoma virus of leopard frog, *Rana pipiens*) Lucké (103, 104, 105); Schlumberger and Lucké (133).

FAMILY IV. ERRONACEAE HOLMES (74)

Viruses of the Encephalitis Group, inducing diseases mainly characterized by effects on nervous tissue.

Key to the genera of family **Erronaceae**

I. Viruses of the typical encephalitis Group affecting man and animals.
1. Genus I. *Erro* Holmes (74).

II. Viruses of the human poliomyelitis Group.
2. Genus II. *Legio* Holmes (74).

III. Viruses of the animal poliomyelitis Group, resembling the lesions of the disease in man, but unrelated.
3. Genus III. *Theilerella* gen. nov.

IV. Viruses of the lymphocytic choriomeningitis Group.
4. Genus IV. *Armlillia* gen. nov.

V. Viruses of the rabies Group.
5. Genus V. *Formido* Holmes (74).

Genus I. Erro

Viruses of the typical Encephalitis Group, inducing diseases mainly characterized by injuries to cells of the brain. Vectors of some known to be ticks; dipterous insects may also transmit. Generic name from Latin *erro*, a vagrant.

The type species is *Erro scoticus* Holmes (74).

Key to the species of genus Erro

I. Affecting sheep principally, but also man.
1. *Erro scoticus* Holmes (74). (Louping-ill virus).

II. Affecting man principally.
2. *Erro silvestris* Holmes (74). (Russian spring–summer encephalitis virus).
3. *Erro japonicus* Holmes (74). (Japanese B encephalitis virus, subsp. *japonicus*. Subsp. nov. *murray valley* encephalitis virus). French (57); Anderson (2); Smithburn (141).
4. *Erro nili* Holmes (74). (West nile virus infection), see also Smithburn *et al.* (145).
5. *Erro scelestus* Holmes (74). (St. Louis encephalitis virus).
6. *Erro ilheus* sp. nov. (South American Ilheus encephalitis virus). Laemert and Hughes (98).

III. Affecting the horse principally, but also man.
7. *Erro equinus* Holmes (74). (Western type infection virus). Meyer, Haring, and Howitt (115); Hammon, Reeves, and Galindo (68).
8. *Erro tenbroeckii* sp. nov. (Equine encephalomyelitis virus). (Eastern type infection virus). Fothergill *et al.* (52); Webster and Wright (169); Ten Broeck and Merrill (156); Giltner and Shahan (60); Getting (59); Feemster and Getting (49).
9. *Erro venezuelensis* sp. nov. (Venezuelen equine encephalitis virus). Beck and Wyckoff (10); Kubes and Ríos (96); Kubes and Diamante (95); Randall and Mills (120).

IV. Affecting the fox.
10. *Erro vulpis* comb. nov. Holmes (74). *Tarpeia vulpis* Holmes (74).

V. Affecting the chicken.
11. *Erro gallinae* comb. nov. Packer, see Merchant (114) (Avian encephalomyelitis virus of chickens). Rhodes (125); *Legio gallinae* Packer, see Merchant (114).

VI. Affecting the horse, cow, and sheep.
12. *Erro bornensis* (Borna-disease virus). Holmes (74).

Genus II. Legio

Viruses primarily causing damage to ganglionic nerve cells of the medulla and spinal cord of man manifest by varying degrees of paralysis, either transient or permanent in character. Transmissible to higher mammals. Certain strains may be adapted to rodents by repeated intracerebral passage. Often recoverable from the feces of infected hosts.

Can be cultivated in tissue cultures of human or monkey fibroblast cells causing cytopathogenic effects. Cannot be readily propagated in the embryonated hen's egg. Nonhemagglutinating. Approximate size by electron microscopy, 20 mμ.

Generic name from Latin *legio*, an army or legion.

The type species is *Legio debilitans* Holmes (74).

Key to the species of the genus **Legio**

Single species, *Legio debilitans* Holmes (74). Comprising three subspecies, Brunhilde I, Lansing II, and Leon III, Rhodes (125); Bodian, Morgan, and Howe (19); Kessel and Pait (85, 86).

Subspecies *debilitans.* Of a group of 100 strains of poliomyelitis virus examined immunologically, 85 were found to belong to type Brunhilde, 12 to subspecies Lansing, and three to subspecies Leon. See Committee on Typing, National Foundation for Infantile Paralysis (31).

Subspecies *lansing.* Armstrong (5, 6); Kessel, Moore, and Pait (83); Type MEF_1 Schlesinger, Morgan, and Olitsky (132); Type Phillips, Kessel, Pait, and Kokko (87); Type Yale or YSK, Trask, Vignec, and Paul (165); Type Wallingford, Trask and Paul (164); Type RCN Barracks, Clark and Rhodes (28). (See also Committee on Typing 1951 (31)).

Subspecies *leon.* Kessel and Pait (84, 86); Kessel, Moore, and Pait (83); Bodian (18). Type Farabauch, Howe and Bodian (See Committee on Typing (1951) (31)). Type Petry, Bodian (See Committee on Typing 1951 (31)).

Genus III. **Theilerella**

Derived from the name of Max Theiler. Viruses causing spontaneous encephalomyelitis in animals. Simulating in character the histological lesions of human poliomyelitis, but exhibiting no apparent immunological or etiological relationship with the human disease.

The type species is *Theilerella muris* comb. nov.

Key to the species of the genus **Theilerella**

I. Affecting the mouse.

1. *Theilerella muris* comb. nov. Holmes (74). (Type TO type species) Theiler (157, 158); Rhodes (125). *Legio muris* Holmes (74). (Type FA) Theiler and Gard (159).

II. Affecting the pig.

2. *Theilerella tescheni* sp. nov. Klobouk (90); Lentz (100); Gard (58); Frauchiger and Hofman (56); Rhodes (125).

Genus IV. **Armlillia***

Genus derived from the names of Armstrong and Lillie (7), commemorating their discovery of the virus of acute lymphocytic choriomeningitis. Viruses principally affecting lower animals, occasionally communicable to man causing clinical signs and symptoms referable to involvement of the central nervous system. Pathology in man not studied.

Type species is *Armlillia erebea* comb. nov. *Legio erebea* Holmes (74).

Key to the species of genus **Armlillia**

I. Affecting mice and man.

1. *Armlillia erebea* comb. nov. Holmes (74). (Lymphocytic choriomeningitis virus). *Legio erebea* Holmes (74). See also Rhodes (125).
2. *Armlillia simulans* comb. nov. Holmes (74). (Pseudo lymphocytic choriomeningitis virus). *Legio simulans,* Holmes (74). See also Rhodes (125).

Genus V. **Formido**

Viruses of the Rabies Group, inducing diseases characterized by involvement of the nervous system only. Generic name from Latin *formido,* a frightful thing.

Type species is *Formido inexorabilis* Holmes (74).

* *Here is an instance where the contributions of a joint authorship have been commemorated in a single patronym. See Recommendation 27 (C) 3. (1).*

Key to the species of the genus **Formido** gen. nov.

I. Affecting the man and animals, carnivora and herbivora.
1. *Formido inexorabilis* Holmes (74). (Rabies virus).

II. Affecting swine.
2. *Formido aujeszkyii* Ansel (4). (Pseudorabies virus of swine, Aujeszky's disease). Syn. *Scelus suillum* Packer. See Merchant (114).

FAMILY V. SMITHBURNIACEAE FAM. NOV.

Viruses first isolated in the forests of Africa and South America, by intracerebral injection mice. Less frequently recovered from blood of man, rodents, and primates.

Key to the genera of family **Smithburniaceae**

I. Viruses isolated from the blood of man and from mosquitoes.
Genus I. *Smithburnia* gen. nov.

II. Viruses isolated from rodents, primates, and mosquitoes.
Genus II. *Jungeblutia* gen. nov.

III. Viruses isolated from mosquitoes alone.
Genus III. *Rocaea* gen. nov.

Genus I. Smithburnia

Generic name derived from the name of Smithburn commemorating his studies in this group of viruses on the Continent of Africa.

Viruses isolated from the blood of man or from mosquitoes, or from rodents causing neurotropic and sometimes viscerotropic lesions in animals. Although isolated from man, the pathogenesis of these agents is imperfectly understood.

The type species is *Smithburnia bunyamwera* comb. nov.

Key to the species of the genus **Smithburnia**

I. Viruses isolated from human blood or mosquitoes.
1. *Smithburnia bunyamwera* comb. nov. Ansel (4). (Bunyamwera encephalitis virus). *Erro bunyamwera* Ansel (4). Smithburn, Haddow, and Mahaffy (144).
2. *Smithburnia bwamba* comb. nov. Ansel (4). (Bwamba fever virus). *Erro bwambae*, Ansel (4); Smithburn, Mahaffy, and Paul (147).

Genus II. Jungeblutia

Viruses recovered from the central nervous system and other tissues of rodents and primates, causing encephalomyocarditis in these species. Viruses causing hemagglutination of sheep erythrocytes Olitsky and Yager (117); Dick (40). Generic name derived from the name of Jungeblut.

The type and only recognized species is *Jungeblutia colombia* sp. nov.

1. *Jungeblutia colombia* (Colombia SK-MM). Jungeblut and Sanders (82); Jungeblut and Dalldorf (81), identical with MENGO virus of Dick (39); Dick, Best, Haddow, and Smithburn (42) and encephalomyocarditis virus (EMC) of Helwig and Schmidt (70); Schmidt (134); Warren, Smadel, and Russ (168). See also Dick, Kitchen and Haddow (44).

Genus III. Rocaea

Viruses isolated from mosquitoes only. Pathogenic to mice and other rodents. Viruses not isolated from man. Generic name derived from the name of Roca-Garcia and commemorating his researches in Eastern Colombia.

The type species is *Rocaea alpha* sp. nov.

Key to the species of genus **Rocaea**

I. Isolated in South America.
1. *Rocaea alpha* sp. nov. (Anopheles A virus). Recovered from two lots of *Anopheles boliviensis* in Eastern Colombia by Roca-Garcia (126).
2. *Rocaea beta* sp. nov. (Anopheles *B* virus). Recovered from same source as *Rocaea alpha*, Roca-Garcia (126).
3. *Rocaea wyeomyia* sp. nov. (Wyeomyia or Sabething I virus). Isolated from *Wyeomyia melanocephala*, in Eastern Colombia by Roca-Garcia (126).

II. Isolated in California.

4. *Rocaea hammonii* sp. nov. (Californian Hammon-Reeves virus). Isolated from *Aedes dorsalis* and *Culex tarsalis* in California. Hammon and Reeves (67).

III. Isolated in Africa.

5. *Rocaea semliki* Ansel (4) comb. nov. (*Erro Semliki*) Ansel (4). Virus isolated from *Aedes abnormalis* in the Semliki Forest of Western Uganda by Smithburn and Haddow (142); Smithburn, Mahaffy, and Haddow (146).
6. *Rocaea dickii* sp. nov. (Uganda S. virus). Isolated from *Aedes* mosquitoes in the Bwamba Country of Uganda by Dick and Haddow (43).
7. *Rocaea zika* sp. nov. Virus isolated from *Aedes africanus* mosquito in the Zika forest of Uganda by Dick, Kitchen, and Haddow (44); Dick (41).
8. *Rocaea ntaya* sp. nov. (Ntaya virus). Isolated from several species of mosquitoes captured in the Ntaya swamp of the Bwamba Country of Uganda by Smithburn and Haddow (143).

FAMILY VI. DALLDORFIACEAE FAM. NOV.

(Enteroviraceae (nomen provisorium) Dalldorf (36))

Viruses commonly found in the feces of infected persons and healthy carriers. Cause of herpangina and pleurodynia in man. There is a single genus.

Genus I. **Dalldorfia** gen. nov.

The name commemorates the researches of Dalldorf (36) and Dalldorf and Sickles (37) on coxsackie disease. The type and only recognized species is *Dalldorfia coxsackie* sp. nov. Divisible into two subspecies, *Coxsackie* and *alpha* on the basis of clinical symptomatology and pathogenicity to man, immature mice, and hamsters.

The type species is *Dalldorfia coxsackie* sp. nov.

Key to the species and subspecies of genus **Dalldorfia**

Viruses isolated from human cases of Coxsackie Disease.

I. *Dalldorfia coxsackie* (Coxsackie disease virus).

1. Subspecies nov. *Coxsackie* comprising 10 different serological varieties tabulated as follows according to Dalldorf (36).

Groups	Type	Representative strain		Reference
		Name	Numbers	
A	1	TT	48249	Dalldorf (36)
A	1	Yale-Easton (2)	48249	Melnick and Kaplan (110)
A	1	B-j	48249	Rowen and Irons (129)
A	2	FL	49190	Dalldorf (36)
A	3	J.Oe.	49191	Dalldorf (36)
A	4		50246	Dalldorf (36)
A	4	Minnesota SY-1	50246	Slater and Syverton (140)
A	4	Minnesota Sy-2	50246	Slater and Syverton (140)
A	4	Yale-Texas	50246	Melnick (109)
A	4	Yale High point	50246	Melnick (109)
A	4	Dallas M-B	50246	Sulkin *et al.* (150)
A	5	Polk	5128	Howitt and Benefield (77) Dalldorf (36)
A	6	C.G.	5011	Dalldorf (36)
A	7	W.P.	50140	Dalldorf (36)
A	8	C.D.	5010	Dalldorf (36)
A	9	P.B.	50546	Dalldorf (36)
A	10	N.K.	50548	Dalldorf (36)

2. Subspecies nov. *alpha* comprising three distinct serological types as follows according to Dalldorf (36).

Groups	Type	Representative strain		Reference
		Name	Numbers	
B	1	P.O.	49683	Dalldorf (36)
B	1	Yale-Connecticut (5)	49683	Curnen *et al.* (33)
B	2	Ohio (RED)	50207	Melnick *et al.* (112)
B	3	Nancy	50531	Melnick and Ledinko (111)
B	3	Washington 518	50531	Lazarus *et al.* (99)
B	4	J.V.B.	51196	Dalldorf (36)
B	4	Powers	51196	Cheever *et al.* (27)

FAMILY VII. REEDELLACEAE FAM. NOV.

Viruses causing generalized systemic infection characterized by fever and necrosis of tissue in the absence of obvious macule, papule, or vesicle formation or of conspicuous involvement of nerve cells.

Key to the genera of family **Reedellaceae**

I. Viruses causing jaundice in man and animals.
Genus I. *Reedella* nomen nov. syn. *Charon* Holmes (74).

II. Viruses of sand-fly fever group.
Genus II. *Sabinia* gen. nov.

III. Viruses of the influenza group.
Genus III. *Tarpeia* Holmes (74).

IV. Viruses of the hog-cholera group.
Genus IV. *Tortor* Holmes (74).

Genus I. **Reedella**

Name derived from Walter Reed, commemorating historic studies on yellow fever. Viruses causing generalized systemic infection in man and animals. Exhibiting a high degree of affinity for liver parenchyma. Often accompanied by the appearance of abnormal bile products in blood and urine with varying degrees of clinical jaundice.

The type species is *Reedella evagatus* comb. nov.

Key to species of the genus **Reedella**

I. Affecting man and animals. Vectors mosquitoes.
1. *Reedella evagatus* Holmes (74) comb. nov. (Yellow-fever virus). *Charon evagatus* Holmes (74). For illegitimate use of Charon, see Becker (13). Type 17D. *Asibi* strain, egg embryo adapted yellow-fever virus vaccine. Sawyer *et al.* (131).
2. *Reedella vallis* comb. nov. (Rift Valley fever virus). *Charon vallis.* Holmes (74).

II. Affecting man alone.
3. *Reedella triginta* sp. nov. (Infectious hepatitis virus). Derived from the Latin word for thirty, suggestive of incubation period of approximately thirty days. Voegt (167); MacCallum and Bradley (107); Havens *et al.* (69).
4. *Reedella centum* sp. nov. (Serum hepatitis virus). Derived from the Latin for a hundred, suggestive of an incubation period of approximately one hundred days. Findlay and MacCallum (51); Findlay (50); Paul, Havens, Sabin, and Philip (119); MacCallum and Bauer (106); Turner *et al.* (166); Sawyer *et al.* (131).

Genus II. **Sabinia**

Viruses of the sand-fly fever group. Characterized by generalized invasion of tissues and high fever. Transmitted by insect and arthropod vectors. Prevalent in dry and arid climates. Genus derived from name of Albert B. Sabin in recognition of his contributions in bringing order into the dengue and sand-fly group of viruses.

The type species is *Sabinia doerrii* sp. nov.

Key to the species of genus **Sabinia**

I. Affecting man.

1. *Sabinia doerrii* sp. nov. (Sand-fly fever virus). Doerr, Franz, and Taussig (45).
2. *Sabinia ashburnii* sp. nov. (Dengue fever virus). Ashburn and Craig (8); Sabin and Schlesinger (130).
3. *Sabinia coloradensis* sp. nov. (American Mountain fever or Colorado tick fever virus). Toomey (160); Becker (11, 12); Topping, Cullyford, and Davis (161); Koprowski and Cox (93).

The causal agent of Fort Bragg or pretibial fever, Tatlock (154); and Melnick and Paul (113) has now been eliminated from the list of virus diseases, see Alexander *et al.* (1).

Genus III. **Tarpeia** Holmes (74)

Viruses of the Influenza Group, inducing diseases characterized principally by involvement of the respiratory tract. Generic name from Latin *Tarpeia*, name of a Roman maiden who treacherously opened a citadel to an enemy.

The type species is *Tarpeia alpha* Holmes (74). *Tarpeia alpha* comprises three subspecies, *Tarpeia alpha*, *beta*, and *taylorii*.

Key to the species of genus **Tarpeia**

I. Affecting man principally.

1. *Tarpeia alpha*. Subspecies *alpha* syn. *Tarpeia alpha* Holmes (74). Types (PR8) Francis (53); Knight (91, 92); Sharp *et al.* (135). Type monmouth (FM1) A-prime, Rasmussen *et al.* (121). Subspecies *beta*. *Tarpeia beta* Holmes (74). Type (Lee) Francis (54); Knight (91, 92); Sharp *et al.* (135). Subspecies *taylorii* (Influenza C virus of Taylor 155). See also Francis *et al.* (55).
2. *Tarpeia premens* Holmes (74). Common cold.
3. *Tarpeia hillemanii* sp. nov., virus isolated from cases of acute respiratory disease, designated RI-67 virus by Hilleman. Hilleman and Werner (72).

II. Affecting pig.

4. *Tarpeia shopei* sp. nov. (Swine influenza). Shope (136).

III. Affecting cat.

5. *Tarpeia felis* Holmes (74). (Feline enteritis or cat distemper.)

IV. Affecting dog.

6. *Tarpeia canis* Holmes (74). (Canine distemper virus).

V. Affecting ferret.

7. *Tarpeia viverrae* Holmes (74). (Ferret distemper virus).

VI. Affecting bird.

8. *Tarpeia avium* Holmes (74). (Laryngotracheitis virus).
9. *Tarpeia pulli* Packer, see Merchant (114). (Infectious bronchitis virus of chickens).

VII. Affecting cow.

10. *Tarpeia vitulae* Holmes (74). (Bovine pneumonenteritis virus).

VIII. Affecting horse.

11. *Tarpeia caballi* Packer, see Merchant (114). (Equine influenza).

Genus IV. **Tortor**

Viruses of the Hog Cholera Group, inducing diseases characterized by involvement of many tissues. Generic name from Latin *Tortor*, tormentor.
The type species is *Tortor suis* Holmes (74).

Key to the species of genus **Tortor**

I. In mammals.
  A. Infecting swine.
    1. *Tortor suis* Holmes (74) (Hog cholera virus).
  B. Infecting cattle.
    2. *Tortor bovis* Holmes (74) (Rinderpest virus).
  C. Infecting horses.
    3. *Tortor equorum* Holmes (74) (African horse sickness virus).
    4. *Tortor equae* Holmes (74) (Mare abortion virus).
  D. Infecting sheep.
    5. *Tortor ovis* Holmes (74) (Blue tongue virus).
  E. Infecting cats.
    6. *Tortor felis* Holmes (74) (Feline enteritis and agranulocytosis virus).
  F. Infecting dogs.
    7. *Tortor vulpis* Holmes (74) Packer, see Merchant (114). (Canine hepatitis virus).
II. In birds.
    8. *Tortor galli* Holmes (74) (Fowl plague virus).
    9. *Tortor furens* Holmes (74) (Avian Newcastle disease virus).

FAMILY VIII. TRIFURACEAE HOLMES (74)

Viruses of the Infectious Anemia Group, inducing diseases mainly characterized by disturbances in balance of blood cells. There is a single genus.

Genus I. **Trifur** Holmes (74).

With characteristics of the family generic name from Latin *trifur*, arrant thief.
The type species is *Trifur equorum* Holmes (74).

Key to the species of genus **Trifur**

I. Affecting the horse.
    1. *Trifur equorum* Holmes (74) (Equine infectious anemia virus).
II. Affecting the fowl.
    2. *Trifur gallinarum* Holmes (74) (Fowl leucosis virus).

FAMILY IX. RABULACEAE HOLMES (74)

Viruses of the Mumps Groups, characterized in general by a special affinity for tissues of the salivary glands. There is a single genus.

Genus I. **Rabula** Holmes (74)

With characteristics of the family. Generic name from Latin *rabula*, pettifogger.
The type species is *Rabula inflans* Holmes (74).

Key to the species of the genus **Rabula**

I. Affecting man.
    1. *Rabula inflans* Holmes (74) (Human mumps virus).
II. Affecting guinea pig.
    2. *Rabula levis* Holmes (74) (Guinea pig salivary gland virus).
III. Affecting hamster.
    3. *Rabula innocuus* Holmes (74) (Hamster salivary gland virus).
IV. Affecting rat.
    4. *Rabula exiguus* Holmes (74) (Rat salivary gland virus).
V. Affecting mouse.
    5. *Rabula latens* Holmes (74) (Mouse salivary gland virus).

Summary of Proposals

Increasing pressure for definitive action on virus nomenclature, as stated by Buchanan (22), has prompted the author to revise Holmes's classification of animal viruses by continued application of the Linnaean system of binomial nomenclature. The present revision of Suborder III, Zoophagineae Holmes (74) has been undertaken and extended by the creation of four new families, eight new genera, thirty-one new species, nine new subspecies, and the proposal of sixteen new combinations. Details of new additions are as follows:—

I. Four new families are proposed:

Family III. *Molitoraceae*, viruses associated with the formation of warts and other proliferative tissue changes.
Family V. *Smithburniaceae*, viruses isolated from the blood of man, mosquitoes, and certain rodents, pathogenic to mice.
Family VI. *Dalldorfiaceae*, viruses isolated from the stools of patients suffering from coxsackie disease.
Family VII. *Reedellaceae*, viruses of the yellow-fever and jaundice producing group.

II. Eight new genera have been proposed as follows:

Morbillifex (Human measles group).
Theilerella (Viruses causing spontaneous encephalomyelitis in animals).
Armlillia (Acute lymphocytic choriomeningitis group).
Smithburnia (Viruses isolated in Africa from human and animal blood).
Jungeblutia (Viruses isolated from the central nervous system and other tissue of rodents and primates causing encephalomyelitis).
Rocaea (Viruses recovered from mosquitoes and pathogenic to rodents).
Dalldorfia (Viruses isolated from the alimentary tract of man or from sewage and flies pathogenic to suckling mice).
Sabinia (Sand-fly fever and related viruses).

III. Thirty-one new species are proposed:

Borreliota meleagridis	(Turkey pox virus)
Borreliota fringillae	(Canary pox virus)
Borreliota columbae	(Pigeon pox virus)
Borreliota jenneri	(Cow pox virus)
Borreliota cuniculi	(Rabbit pox virus)
Briareus torresii	(Alastrim or Kaffir pox virus)
Briareus zosticus	(Zoster or Shingles virus)
Morbillifex embryorum	(Rubella virus)
Morbillifex subitus	(Roseola subitum or Sixth disease virus)
Molitor fibromatis	(Fibroma virus of Shope)
Molitor luckei	(Adenocarcinoma virus of leopard frog)
Erro ilheus	(South American Ilheus virus)
Erro tenbroeckii	(Eastern equine encephalomyelitis virus)
Erro venezuelensis	(Venezuelan encephalitis virus)
Theilerella tescheni	(Teschen disease virus)
Jungeblutia colombia	(Columbia SK-MM; mengo virus of Dick, and encephalomyocarditis virus of Helwig and Schmidt)
Rocaea alpha	(South American Roca-Garcia A virus)
Rocaea beta	(South American Roca-Garcia B virus)
Rocaea wyeomyia	(South American Roca-Garcia Wyeomyia virus)
Rocaea hammonii	(California Hammon-Reeves virus)
Rocaea dickii	(Uganda S virus of Dick)
Rocaea zika	(Zika virus of Dick)
Rocaea ntaya	(Ntaya virus)
Dalldorfia coxsackie	(Coxsackie virus)
Reedella triginta	(Infectious hepatitis virus)
Reedella centum	(Serum hepatitis virus)
Sabinia doerrii	(Sand-fly fever virus)
Sabinia ashburnii	(Dengue fever virus)
Sabinia coloradensis	(American mountain fever virus)
Tarpeia hillemanii	(Primary atypical pneumonia virus of Hilleman)
Tarpeia shopei	(Swine influenza)

IV. Nine new subspecies have been proposed:

Erro japonicus subsp. *murray valley*	(Murray Valley encephalitis virus)
Legio debilitans subsp. *debilitans*	(Poliomyelitis Brunhilde virus)
Legio debilitans subsp. *lansing*	(Poliomyelitis Lansing virus)
Legio debilitans subsp. *leon*	(Poliomyelitis Leon virus)
Dalldorfia coxsackie subsp. *coxsackie*	(Coxsackie virus A)
Dalldorfia coxsackie subsp. *beta*	(Coxsackie virus B)
Tarpeia alpha subsp. *alpha*	(Influenza A virus)
Tarpeia alpha subsp. *beta*	(Influenza B virus)
Tarpeia alpha subsp. *taylorii*	(Influenza C virus)

V. Sixteen new combinations are proposed:

Borreliota myxomatis	(*Molitor myxomae* Holmes (74))	Rabbit myxoma virus
Borreliota ovina	(*Variola ovina* Blaxall (17))	Sheep pox virus
Borreliota marmorans	(*Scelus marmorans* Holmes (74))	Mouse pox virus
Briareus variolae	(*Borreliota variolae* Holmes (74))	Small pox virus
Morbillifex morbillorum	(*Briareus morbillorum* Holmes (74))	Measles virus
Hostis ecthymatis	(*Borreliota ecthymatis* Merchant (114))	Contagious ecthyma of sheep virus
Erro vulpis	(*Tarpeia vulpis* Holmes (74))	Fox encephalitis virus
Erro gallinae	(*Legio gallinae* Holmes (74))	Avian encephalomyelitis virus
Theilerella muris	(*Legio muris* Holmes (74))	Theiler's TO mouse encephalitis virus
Armlillia erebea	(*Legio erebea* Holmes (74))	Lymphocytic choriomeningitis virus
Armlillia simulans	(*Legio simulans* Holmes (74))	Pseudo lymphocytic choriomeningitis virus
Smithburnia bunyamwera	(*Erro bunyamwera* Ansel (4))	Bunyamwera encephalitis virus
Smithburnia bwamba	(*Formido bwambae* Ansel (4))	Bwamba fever virus
Rocaea semliki	(*Erro semliki* Ansel (4))	Semliki Forest virus
Reedella evagatus	(*Charon evagatus* Holmes (74))	Yellow fever virus
Reedella vallis	(*Charon vallis* Holmes (74))	Rift Valley fever virus

VI. One new name has been substituted, *Reedella* for genus *Charon* Holmes (74) and the specific epithet *myxomatis* for *molitor myxomae* Holmes (74).

Attention is drawn to the existence of four genetic and specific epithets which have been proposed in the past, but have been overlooked in subsequent literature.

Borreliota ovina	(Blaxall (17))	Sheep pox virus
Borreliota caprina	(Blaxall (17))	Goat pox virus
Borreliota suilla	(Blaxall (17)), *Borreliota suis* Holmes (74))	Swine pox virus
Borreliota equina	(Blaxall (74))	Horse pox virus
Buistia paschenii	(Mackie and van Rooyen (108))	Variola–vaccinia virus

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Contents

Page

Yeasts Occurring on Apples and in Apple Cider—*D. S. Clark, R. H. Wallace, and J. J. David* - - - - - - - - - - - - - - - 145

A Standard Inoculum for Citric Acid Production in Submerged Culture—*R. Steel, S. M. Martin, and C. P. Lentz* - - - - - - - - - - 150

Studies on the Propagation of Influenza and Mumps Viruses in Tissue Culture with Chemically-defined Media—*M. M. Burr, M. E. Campbell, J. F. Morgan, and F. P. Nagler* - - - - - - - - - - - - - - 158

Stages in the Development of Two Insect Viruses— *F. T. Bird and Mary M. Whalen* - - - - - - - - - - - - - - - - 170

Antibacterial Action of a Reaction Product of Cysteine and Iron. I. Development of the Substance in Media for *Mycobacterium tuberculosis*—*Jack Konowalchuk, N. A. Hinton, and G. B. Reed* - - - - - - - - - 175

Antibacterial Action of a Reaction Product of Cysteine and Iron. II. Preparation and Properties of the Substance—*Jack Konowalchuk, J. C. Clunie, N. A. Hinton, and G. B. Reed* - - - - - - - - - - - - - 182

Studies on the Nutrition of *Helminthosporium sativum* and Certain Related Species—*E. A. Peterson and H. Katznelson* - - - - - - - - - 190

The Metabolism of Seven-carbon Sugars, Acids, and Alcohols by Bacteria and Yeasts—*W. B. Moore, A. C. Blackwood, and A. C. Neish* - - - - - - 198

Biological Oxidation of Naphthalene—*R. J. Strawinski and R. W. Stone* - - 206

Antibacterial Action of a Reaction Product of Cysteine and Iron. III. *In vivo* Action on Pneumococcus Infection in Mice—*N. A. Hinton, Jack Konowalchuk, and G. B. Reed* - - - - - - - - - - - - - - - 211

A Cytological Study of the Lysogenization of *Shigella dysenteriae* with P1 and P2 Bacteriophages—*J. F. Whitfield and R. G. E. Murray* - - - - - 216

A Revision of Holmes's Classification of Animal Viruses, Suborder III (Zoophagineae)—*C. E. van Rooyen* - - - - - - - - - - - 227